A Simple Preparative Polyacrylamide Gel Electrophoresis for the Purification of Chymotrypsin Inhibitor Isoforms from *Ganoderma lucidum*

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Protein protease inhibitors are widely distributed in biological world[1-3] and believed to be involved in controlling functions in a variety of fundamental physiological proteolytic processes.[4-6] Some purified exogenous protease inhibitors are found beneficial in regulating certain disease states where proteolytic enzymes are related to pathological situations.[7-10] In general, protease inhibitors or isoinhibitors have been purified by a number of steps of various chromatographical methods.[11-13] Due to the similarities in their structures and chromatographical properties, the purification of these isoinhibitors is usually a time consuming, tedious work. In this report we described a simple and fast preparative polyacrylamide gel electrophoresis (PAGE) method involving reverse field electroelution for purification of chymotrypsin inhibitor isoforms from a fruit body of *Ganoderma lucidum*. Although the preparative electrophoresis with electroelution has been used for years,[14-17] they usually require a separate preparative electrophoresis kit, dialysis membrane or high salt buffer. Preparative electrophoresis technique described here includes separation of target protein by conventional PAGE in native or denatured state followed by electroelution of the protein of interest from the gel using the same electrophoresis kit with a minor modification. No expensive device or membranes are required. By applying this reverse field electroelution method, chymotrypsin inhibitor isoforms from *Ganoderma lucidum* (GCI) could be easily purified even in one step from the crude extract.

Experimental Section

Polyacrylamide gel electrophoresis (PAGE). The reagents and gel preparations for PAGE were followed as the method of Laemmli.[18] Electrophoresis was run in native state in a vertical slap gel electrophoresis unit, HSI SE 250 Mighty Small II (Hoefer Scientific Instruments) at 30-35 mA. After termination, one of the glass plates is removed and cut the gel vertically 0.5 cm strip from the both ends and the strips were stained with Coomassie blue. The stained gel strips were then aligned with each side of the remainder gel and the band of interest protein of the slap was excised horizontally with the guide of stained bands. The electoelution was carried out as follows. 4 mL of 5% polyacrylamide (PA) supporting gel was made in a glass plates sandwich and the excised unstained gel strip was placed on top of the supporting gel and fixed by addition of small amount of 5% PA solution. The electrode solution (0.5 M Tris) was placed into the rest of sandwich no higher than to 0.5 cm from the top of the plates (about 3.0 mL). Instead of the anodic electrode equipped with the kit, a new platinum electrode was connected to the anode. Then the red cable was plugged into the black connector on the power supply and black cable into the red connector. Electrophoresis was run at 30 mA for one hour upward instead of usual running down. Figure 1 depicts the summary of electroelution process in reverse field. After elution, all the electrode solution were collected using a loading pipette tip or a syringe and the pH of the solution was adjusted to the optimum pH for the assay. Gel strip was taken out and stained to be certain that all the proteins were eluted out during the run.

Purification of GCI.[19,20] 10 g of dried *Ganoderma lucidum* (Korean cultured, 6 years old) were homogenized in

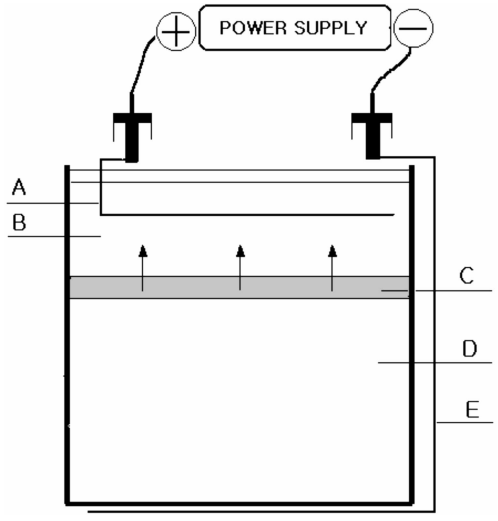

Figure 1. A schematic representation of the reverse field electroelution process: (A) platinum anodic electrode; (B) upper buffer (electrode solution); (C) gel strip containing protein band; (D) supporting gel (5% polyacrylamide gel); (E) cathodic electrode. Arrows indicate the direction of migration.

200 mL of lysis buffer (0.05 M Tris-HCl buffer, pH 7.6, 0.1 M NaCl, 5 mM mercaptoethanol) containing 1 mM PMSF. The homogenate was centrifuged and resulting supernatant was heated at 60 °C for 20 min and centrifuged. The supernatant was fractionated by 30%-60% ammonium sulfate saturation and resulting precipitates were dissolved in a small amount of 0.05 M Tris-HCl buffer (pH 7.6). 5 mL of this crude inhibitor preparation was applied onto a Sephadex G-75 column (1.6 × 80 cm, GradiFrac, Phamacia Biotec.) and proteins were eluted using the equilibration buffer and fractions (4 mL/fraction) were collected at a flow rate of 0.4 mL/min. Fractions corresponding to chymotrypsin inhibitory activity were pooled and concentrated in an Amicon ultrafiltration apparatus using a PM 10 membrane (Amicon, Beverly, MA. USA).

250 μL of concentrated sample from gel filtration chromatography was applied onto a UNO-Q1 column (1 × 5 cm), in fast protein liquid chromatography (Biologic HR, Bio-Rad). Bound proteins were eluted by a linear gradient of ionic strength (0-1 M NaCl in 0.05 M Tris buffer, pH 7.6). Fractions (0.3 mL/fraction) were collected at a flow rate of 0.3 mL/min and those corresponding to chymotrypsin inhibitory activity were pooled and concentrated by ultrafiltration.

Assays of enzyme and inhibitor activity. Chymotrypsin (Sigma, bovine pancreas) activity was assayed according to the modified method of Fritz *et al.*.[21] Standard assay mixture contained 0.05 M Tris-HCl buffer, pH 7.6, 2×10^{-5} M peptide substrate, N-Suc-Ala-Ala-Pro-Phe-p-nitroanilide and chymotrypsin in 0.5 mL total volume. One unit of enzyme is defined as the amount of enzyme that induces the conversion of 1 μmol substrate/min. The chymotrypsin inhibitory activities were assayed in a similar manner with the addition of the inhibitor to the reaction mixtures. Protein was determined by the method of Bradford[22] using BSA as the standard.

Results and Discussion

The IEC-chromatogram of GCI presents multiple inhibitor active regions indicating that GCI exists in multiple isoforms.[20] Instead of applying additional chromatographic steps, a preparative electrophoresis technique was designed in order to simplify the purification of isoforms of GCI. A conventional analytical electrophoresis unit was used for both separation and elution of proteins from the gel. In the elution step, proteins were mobilized upward in the gel and eventually driven out into the electrode solution in the reversed electric field.

In Figure 2, the result of electroelution of hemoglobin (Sigma, bovine, substrate powder) as a model protein is shown. 0.5 mg hemoglobin was electrophoresed in native state in a 10% PA gel and eluted in reversed field. Since the protein band could be identified in the gel by its own color, the staining step for guidance was not required. At each indicated time of elution period, 200 μL of electrode solution was taken and observed the absorption spectrum at

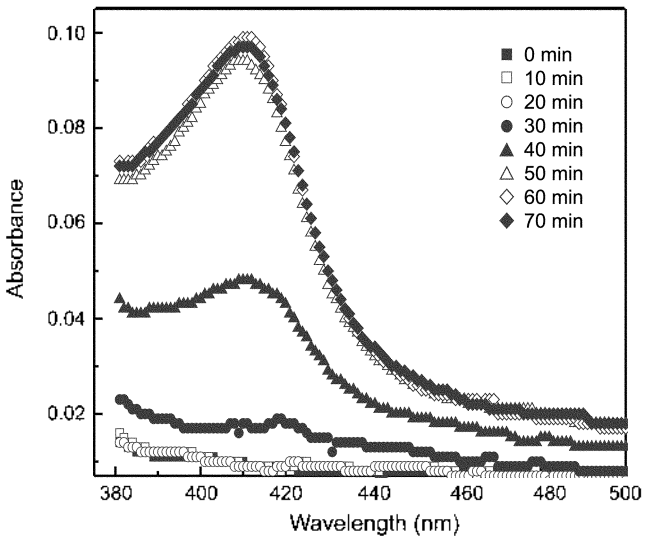

Figure 2. Absorption spectrum of electroeluted hemoglobin at various times during reverse field electroelution.

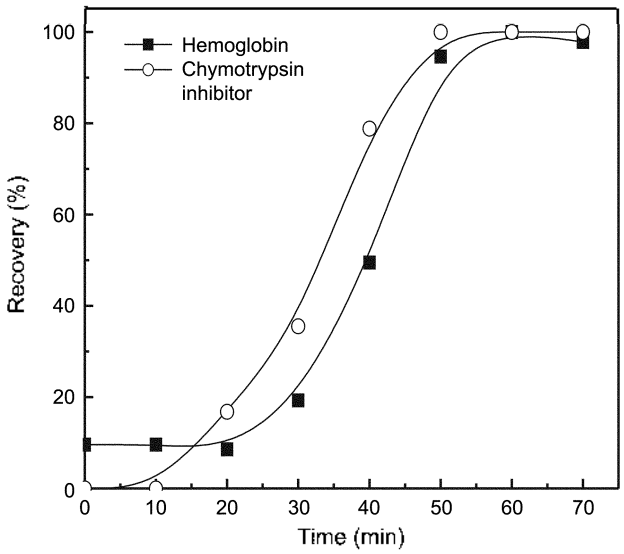

Figure 3. The efficiences of electroelutions of hemoglobin and chymotrypsin inhibitor. At indicated times, recoveries were determined by measuring A_{410} (hemoglobin) and chymotrypsin inhibitory activities (for chymotrypsin inhibitor) of the electrode solution.

380-500 nm and then returned back to the electrode solution. Most of the proteins were eluted in between 40-60 min. The elution pattern of pure chymotrypsin inhibitor (Sigma, soybean) was compared to that of hemoglobin (Figure 3). The recoveries were determined by comparing the A_{410} of hemoglobin eluate or chymotrypsin inhibitor activity of electrode solution at indicated times to the respective control solutions. Under the standard condition, two proteins follow similar elution paths and the recoveries were quantitative (>98%) at the end of 1-hour elution for both model proteins.

Since a limited amount of upper buffer is used in the reverse field electroelution, the progressive decrease of pH ensues in the upper buffer (electrode solution) during electroelution, and thus prevents the eluting out of protein

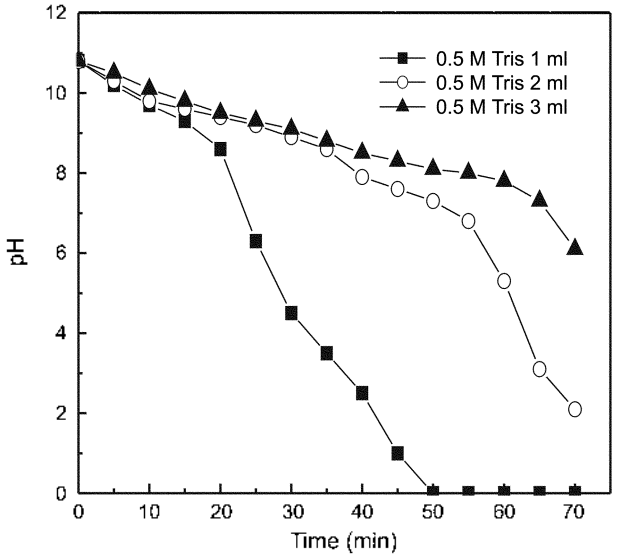

Figure 4. pH changes in three different volumes of upper buffer solution during reverse field electroelution in HIS SE 250 Mighty Small II.

into the electrode solution.[23-25] To overcome this problem, basic 0.5 M Tris solution was chosen as the electrode solution. In Figure 4, the pH changes of this electrode solution in three different volumes during elution are shown. In 3 mL electrode solution, pH varied from 8.4 to 7.8 during 40 min-60 min of elution period, during which, most of proteins are eluted.

In our preliminary purification of GCI by FPLC-IEC and PAGE, four chymotrypsin inhibitor isoforms, namely GCI-I, II, III, and IV, as numbered in the order of increased retention time in the IEC- chromatography could be identified. Inhibitor fractions, GCI-I and GCI-II were further purified by reverse field electroelution and the purity of the final inhibitor fractions were confirmed by the single peaks in the reverse-phase HPLC (Waters, M996 GPC system). The molecular weights of these purified inhibitor isoforms were determined to be 17,585 Da and 17,565 Da for GCI-I and GCI-II, respectively, by MALDI-TOF analyses. The rest of GCI isoforms were not completely purified yet.

In Table 1, the purification details of GCIs by chromatography and also by direct preparative electrophoresis from the crude extract are shown. Besides the timesaving, one-step purification enhanced the specific activities of purified inhibitor isoforms about 3 folds as compared to chromatographic purification. As a rough approximation, if we assume the even distribution of four GCI isoforms in *Ganoderma lucidum* extract, the recoveries of GCI isoforms in one step preparative electrophoresis can be calculated as 41% and 34% for GCI-I and GCI-II, respectively. Sa'-Pereira *et al.*[17] applied the preparative electrophoresis to the purification of xylanase from *Bacillus* sp and reported the recovery of less than 5% of enzyme activity from ammonium sulfate precipitate. Recently Radko *et al.*[23] developed a new electroelution method of direct elution orthogonal to the direction of migration showing that six different model proteins worked with the recoveries from 25% to 33% depending on the proteins.

Preparative PAGE had been used for years in order to simplify the purification of protein. Although several different types of preparative electrophoresis cells have been available, they are difficult to set up and are often leak besides they take long time. The gel excision and elution method usually requires dialysis membrane or very high salt solution to receive the eluted protein.[26,27] Certain proteins may be adsorbed by membrane and some present solubility problem in using very high salt concentration. The preparative electrophoresis method described in this article uses an analytical vertical slap gel kit, which is used routinely in every lab in the world. Just simple modifications of reversing the electric field and using a new electrode (anode) are sufficient for a fast and efficient elution of protein from gel. By selecting the basic electrode solution, proteins in the gel can be eluted quantitatively even into a small volume (2-3 mL) of electrode solution.

In conclusion, the reverse field electroelution is fast and reliable method for the purification of protein provided that the protein can be separated and visualized in the gel. This study has proven that this preparative electrophoresis is applicable even to crude extract as well as model proteins. It should be particularly useful to those who want to purify the enzymes or enzyme inhibitor isoforms, which are often difficult to separate by chromatographic method because of the structural similarity and/or their low concentrations.

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Table 1. Purification details of chymotrypsin inhibitor isoforms from *Ganoderma lucidum*

Purification step	Inhibitor Isoforms	Protein (mg)	Activity (Unit)	Specific activity (U/mg)	Yield[a] (%)
Crude extract	GCI-I, II, III, IV	33.80	2275	67.3	100 (400)
Gel filtration + UNO-Q1	GCI-I	0.60	244	409.4	10.7 (42.8)
	GCI-II	0.57	203	355.5	8.9 (35.6)
Reverse Field Electroelution	GCI-I	0.20	231	1172.6	10.2 (40.8)
	GCI-II	0.17	196	1152.9	8.6 (34.4)

[a]Values in parentheses represent the % yield calculated for individual inhibitor isoform on the basis of the even distribution of isoforms of GCI

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