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Article

Separation and Isolation of Fusion Protein using a New Native Preparative PAGE Device

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A human serum albumin and Thymosin α1 (HSA-Tα1) fusion protein was designed and over-expressed in *Pichia pastoris*. To purify the fusion protein, a new native preparative electrophoresis system that involved a modified device with a sample receiving chamber, and an assay method with Coomassie Blue G-250 tracing the collection of the protein of interest. In this device, two gels were run in parallel: native vertical collecting polyacrylamide gel electrophoresis (PAGE) and native vertical tracing PAGE. Samples mixed with or without Coomassie Blue G-250 loading buffer were separately loaded to the two aforementioned gels, and the fractions were collected until the tracing protein band combined with dye reached 1 cm from the sample-receiving chamber at the bottom of the gel. Approximately nine fractions were collected at regular intervals of 15 min. HSA-Tα1 fusion protein with 95% relative homogeneity was harvested and manifested similar immunological activities as synthetic Tα1 after a single-step purification of this preparative PAGE. As a result, this system offers a new, rapid and simple method for the purification of the protein of interest.

Introduction

Thymosin α1 (Tα1) is a biologically active polypeptide with a heat-stable, highly acidic molecule composed of 28 amino acid residues (1). It is well known that Tα1 plays important roles in modulating immune response. Tα1 has been shown to modulate the levels of terminal deoxynucleotidyl transferase activity in thymocytes (2) and to stimulate the production of lymphokines, such as macrophage migration inhibitory factor, interferon, interleukin-2 and interleukin-2 receptor (3–5). Furthermore, there is evidence to suggest that Tα1 may influence the recruitment of pre-natural killer cells that become cytotoxic after exposure to interferon (6–7). More importantly, thymosins may have significant clinical applications. Clinical applications of Tα1 as primary or adjunctive therapy indicate that it may be of potential utility for the treatment of chronic hepatitis B infection, certain forms of cancer, primary immunodeficiency diseases, acquired immunodeficiency disease syndrome (AIDS), vascular biology and cancer pathogenesis (8–9).

Currently, Tα1 is either isolated from calf thymus using a multistage chromatographic purification or obtained by total chemical synthesis (10). A biotechnological approach using the recombinant gene expression in engineering bacteria seems much more promising.

Human serum albumin (HSA), consisting of 585 amino acids, is the most abundant serum protein, with a major function of maintaining an osmotic pressure of plasma and acting as a carrier of endogenous and exogenous molecules (11). HSA is a non-glycosylated protein devoid of enzymatic function with very low renal clearance and a 14–20-day *in vivo* half-life in human beings. The recombinant therapeutic proteins fused to serum albumin are not likely to contribute significantly to the total albumin pool because of the relative abundance of albumin in plasma. Furthermore, HSA may be a suitable carrier for increasing the half-life of small proteins in plasma (12, 13). Hence, a fusion protein HSA-Tα1 was constructed in our laboratory by the recombinant gene expression and applied in this paper.

Over the last 20 years, there have been great advancements in protein synthesis, recombinant organism expression of complex molecules, particularly proteins, and hybridization of cells to produce monoclonal antibodies. These developments have led to an increasing need for apparatus and methods for efficiently separating desired products from co-products and various other contaminants. As a result, it is very important to devise simple and rapid purification techniques to study its biotechnological activity (14).

Electrophoresis is one of the most widely used versatile techniques, both as an analytical technique and as a preparative tool (15). Owing to the high resolving power of electrophoresis, effective separation over a broad mass range from below approximately 10,000 to 250,000 relative molecular mass (Mr) has been achieved (16); particularly for native electrophoresis, separation of protein complexes can be conducted up to the range of 3,000,000 (Mr) (17), therefore, it is frequently deployed for the fractionation from simple organic molecules to macromolecules such as polypeptides, enzymes, blood factors, nucleic acids, polyclonal antibodies (18) and other components of biological interest (17). In previous studies, Fountoulakis applied preparative electrophoresis to separate interferon and interferon receptor complexes for crystallization purposes (19). Additionally, a denatured sample of polipoprotein-B purification was carried by a one-step preparative electrophoresis using the Bio-Rad Model and peaks eluted in preparative electrophoresis were pooled and concentrated by ultrafiltration through a 10,000 (Mr) MWCO (Molecular Weight Cut Off) (20). Hayakawa described a macro-scale preparative electrophoresis device (3.6 cm i.d. disk gel) that was successfully used with sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) for the purification of a highly hydrophobic membrane-bound protein, dipeptidyl peptidase (21). Moreover, native PAGE coupled with a second dimensional SDS–PAGE and mass spectrometry has emerged as a powerful tool for *Streptomyces coelicolor* cytoplasmic protein complexes (22). Further applications for separating urinary glycosaminoglycans in their native forms have been developed (23).

Although a variety of apparatuses are available commercially, they are complex, expensive or difficult to set up (24). In this

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study, a HSA-Tα1 fusion protein was over-expressed in *Pichia pastoris* and purified from the culture with a new preparative electrophoresis apparatus, including a detachable collecting unit, and an assay method with Coomassie Blue dye for tracing protein running in the gel was applied in a native preparative PAGE. The activity of fusion protein was performed *in vitro* by the E-rosette bioassay. The purpose of this paper is to offer a rapid and simple new assay for protein purifying with native preparative electrophoresis.

Materials and Methods

Device construction

A new preparative device was constructed as depicted in Figures 1 and 2. The device is composed of two vertical plate gels: one was applied for collecting the protein of interest; the other was applied for tracing the optimal collective time of protein of interest.

To fix two vertical plate gels and to prevent leakage from the cathode buffer solution, a waterproof plastic mattress was designed, as shown in Figure 2; between the two vertical plates, a condensing chamber was developed for a cooling system and attached to the lowest end of the cathode buffer solution chamber.

In this new electrophoresis device, a detachable sample-receiving chamber was designed in the anode buffer solution chamber, as shown in Figures 2 and 3. With an open upper end for harvesting the protein of interest, the chamber is sealed with a layer of semi-permeable membrane that is adhered to the support framework (Figure 3) composed of macromolecular polyethylene material; moreover, to inlay this detachable chamber, a recess was also designed in this new device (Figure 2).

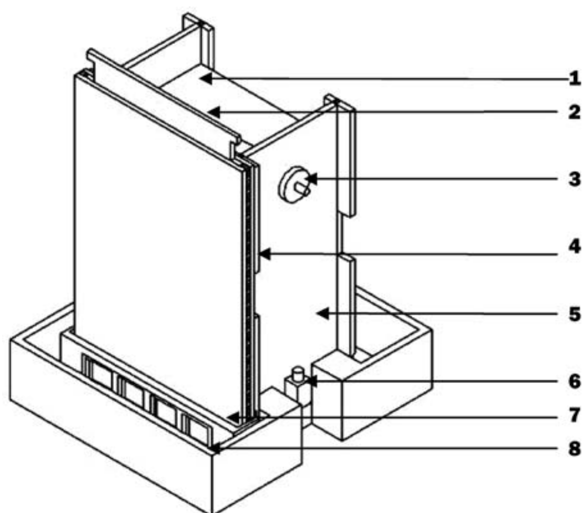

Figure 1. The profile of new modified native preparative electrophoresis device. In here, corresponding number separately represent: 1, cathode buffer solution tank; 2, slots of the comb; 3, condensed water inlet; 4, gel glass plate; 5, the condensation chamber; 6, electrode jack; 7, sample-receiving chamber; 8, anode buffer solution tank.

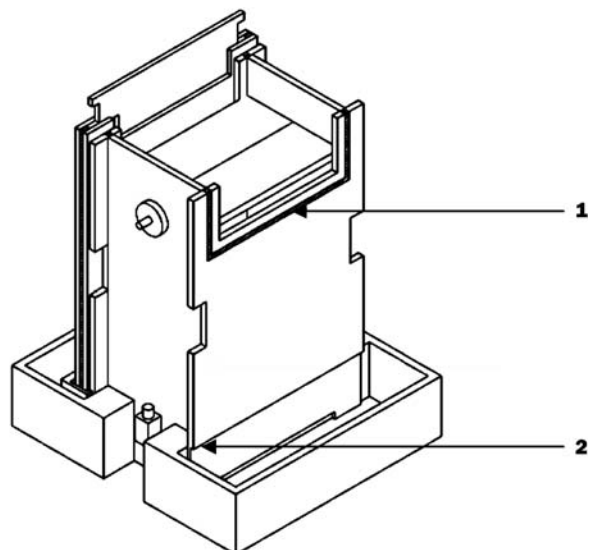

Figure 2. The facial profile of new modified native preparative electrophoresis device. In here, corresponding number separately represent: 1, waterproof plastic mattress which was used for preventing from leakage of electrophoresis buffer. 2, the recess which was designed to inlay the sample-receiving chamber.

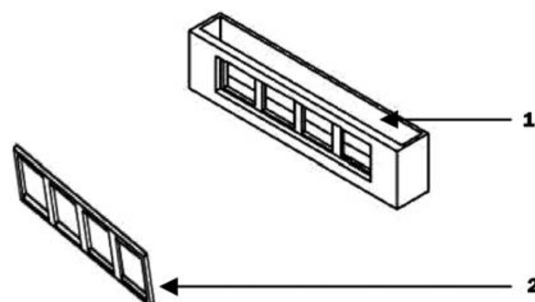

Figure 3. The profile of the sample-receiving chamber. In here, corresponding number separately represent: 1, sample collection in which the interest of sample was collect with syringe. 2, the detachable support framework which was used to fix semi-permeable membrane more conveniently.

Materials

Ammonium persulfate and *N*,*N*′-methylene bisacrylamide were obtained from Scigene (Shanghai, China), and Coomassie Blue G250 was purchased from Amresco (Solon, OH). All other chemicals used in solvents and buffers were of analytical grade and purchased from Wanqing (Nanjing, China).

Sample preparation: High cell density fermentation of Pichia pastoris in fermentor

Fermentation was performed according to the procedure described by Stratton (25). Briefly, starter culture originated from a fresh colony on YPD plate (yeast extract peptone dextrose medium, including 1% yeast extract, 2% tryptone, 1% glucose, w/v) and grew under constant shaking at 30°C until optical density at 600nm (OD600) reached 10–15. The cultures were inoculated with 10 % (v/v) inoculums into a 5-L vessel containing 2.5 L base salt medium (BSM). The pH was

adjusted to 5.0 with 25% ammonia. Agitation was set at 1,200 rpm, and the airflow was maintained at 1–2 vvm to keep the dissolved oxygen (DO) level at least above 30% (v/v). After 72 h, the sample was collected. The fermentation broth was centrifuged with 10,000 × g (−4°C,15 min), and then the supernatant was collected to desalt and stored at −70°C until analysis.

Protein assay

The protein content was determined using the Bradford assay method (26).

Native vertical preparative gel electrophoresis

In this device, two gels run in parallel: native vertical collecting PAGE and native vertical tracing PAGE. The protocol for native PAGE was a modification of Hames non-denaturing PAGE (27). The primary differences from the common system are the absence of SDS and β-mercaptoethanol in electrophoresis, loading buffers and non-boiled samples (27).

An acrylamide–*N,N'*-methylene bisacrylamide solution, 29:1 (3.3% C) was stored at 4°C. The non-colored loading buffer A (5×) used in native vertical collecting PAGE contained 1M Tris adjusted to pH 6.8 with HCl, 50% (v/v) glycerol; loading buffer B (5×) used in native vertical tracing PAGE contained 1M Tris adjusted to pH 6.8 with HCl, 50% (v/v) glycerol and 0.5% (w/v) Coomassie Blue G 250.

Electrophoresis operation

A new electrophoresis apparatus with total length of 24 cm was used, and native stacking and separating gel of 5% T, 3.3% C and 10% T, 3.3% C, respectively, were prepared with 14 cm width and 2–3 mm thickness. Because the device is vertical, samples are easy to load and require no special combs or wells. The sample mixed with the aforementioned buffer A and buffer B was loaded and the electrophoresis run at 4–7°C using a condensation chamber with cooling water. The voltage started at 100 V until the protein samples were within the stacking gel, and then the value was then set at 200 V. The fractions were collected at 15-min intervals until the tracing reached approximately 1 cm from the sample-receiving chamber at the bottom of the gel. Protein elution from the gel was desalted with the ultra-filtration membrane 6,000 Mr MWCO and collected for further analysis.

SDS–PAGE

Fractions eluted in the preparative electrophoresis were pooled. An aliquot of each fraction was mixed with a loading buffer that contained 62.5 mM Tris adjusted to pH 6.8 with HCl, 2% (w/v) SDS, 0.01% (w/v) Bromophenol Blue, 10%(v/v) glycerol and 5% (v/v) β-mercaptoethanol, and then was heated at 100°C for 5 min before loading. A 5% T (w/v) stacking gel and 10% T (w/v) separating gel were prepared and performed in a mini-gel apparatus (Biorad). The gels were stained with Coomassie Brilliant Blue R-250 [0.1% (w/v) in 50% (v/v) methanol–10 % (v/v) acetic acid–water] and destained in the same solvent without the dye to remove background.

Bioactivity assay of Tα1

Lymphocytes from healthy human donors were applied in this assay. After separation by Ficoll centrifugation, T-cells were washed and resuspended in RPMI-1640/FCS (7:3) to 5 × 106 cells/mL. Aliquots of 200 μL were distributed into several tubes. After being blocked in a 45°C thermostatic water-bath for 30 min, the cells were incubated with 100 μL of various concentrations of either synthetic Tα1 or the fusion protein HSA-Tα1 at 37°C for 1 h. To each tube, 200 μL of sheep red blood cell (SRBC) suspension (108 cells/mL) was then added, and the tubes were centrifuged at 150 × g for 3 min and kept at 4°C overnight. Slides were prepared from each tube and the percentage of E-rosette-forming cells was determined by counting 200 lymphocytes (28).The result was analyzed by SPSS statistics software.

Results and Discussion

Two of 1,000-μL samples containing 1.2∼1.4 mg of protein were mixed with 200 μL native loading buffer A and B and loaded into native vertical tracing gel and native vertical collecting gel. From the nine collecting tubes of the fraction, the maximal preparative capacity of the device should be nearly 1.8–2.0 mg/h; approximately 0.4 mg protein of interest (>95% pure with high pressurized liquor chromatography), which was enough to do further bioactivity analysis, was obtained and the result of SDS–PAGE analysis is shown in Figure 4.

The E-receptor on the T-cell is believed to be involved in T-cell activation and adhesion processes, and the addition of optimally active thymosin preparation may restore the erythrocyte rosette-forming capacity (ERFC) of heat-treated lymphocytes. Based upon these findings, the E-rosette bioassay was developed for identifying T-lymphocyte-stimulating compounds (28). Tα1 could restore the erythrocyte rosette-forming capacity of heat-treated or trypsin-treated lymphocytes. In this paper, an evident dose dependence was observed and high dose display significance with blank control ($P < 0.05$). The fusion protein HSA-Tα1 manifested similar immunological activity to synthetic Tα1 (Table I). Moreover, according to the standard technique WS-XG-042-2000-2003 of the State Food and Drug Administration of China (SFDA) (29), the results were also expressed as an indication of increasing the erythrocyte rosette-forming capacity compared with blank control. In Table I, the result of high doses of the fusion protein HSA-Tα1 and synthetic Tα1 have exceeded 10% compared with the blank control, which demonstrates their activity.

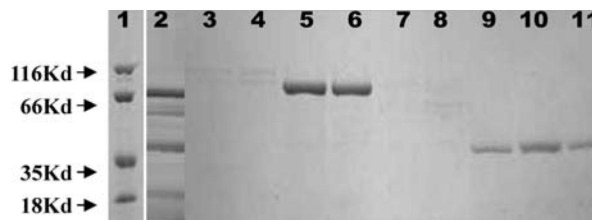

Figure 4. Analysis of fraction by SDS-PAGE (10% T). The lane 1: mark; the lane 2: sample, the lane 5 and 6: purified HSA-Tα1 fusion protein, the rest lane: hybridprsemipermeable membranes.

Table I
Erythrocyte-Rosette Assay Compared to the HSA-Tα1 Fusion Protein with Synthetic Tα1*

	Concentration (μg/mL)	E-rosette increased number
Synthetic Tα1	0.1	40
	0.2	**48**
	0.4	59[†]
HSA-Tα1 fusion protein	2.2	37
	4.4	43
	8.8	**49.5**
Control	0	34

*An E-rosette was defined as a lymphocyte that bound three or more sheep erythrocytes and was determined with a microscope by counting 200 lymphocytes. The assays were repeated three times. Values in bold show that the activity of the synthetic Tα1 and HSA-Tα1 fusion proteins have significance compared with the control ($P < 0.05$) analyzed with SPSS software 10.0.
[†]Mean $P < 0.01$.

Indeed, the Tα1 construct displays a relatively low relative molecular mass (Mr = 3108), a prerequisite for efficient clearance by the kidneys. On the other hand, genetic coupling of small peptides to neutral pharmacological carriers has been proposed to reduce renal filtration by increasing their relative molecular mass above the 50,000 cutoff size (30, 31). An HSA and Interferon-α (HSA−IFNα) fusion protein was reported with an 18-fold longer half-life in the plasma of Cynomolgus monkeys than IFNα. (32). An HSA and cluster of differentiation 4 (HSA−CD4) fusion proteins was found to have a higher elimination of half-life than that of soluble CD4. In particular, it was reported that genetic coupling of soluble CD4 or IFNα to HSA did not abrogate the antiviral properties of the therapeutic protein (31). Hence, the HSA-Tα1 fusion protein is much more promising.

Although some sophisticated electrofractionation systems provide acceptable preparative electrophoresis, some defects exist. For example, commercial apparatuses that use a tube gel with a cooling system for continuous elution electrophoresis are cumbersome with off-column flow-cell detectors, complicated heat removal systems and awkward buffer replenishment systems (24). In the device designed by Lim (33), the protein of interest was detected by the radiolabel with 125I and taken by a pump, and final chromatography on Sephacryl S-200 was necessary to remove SDS and other impurities. Because they are related, their device may require a tedious process for making running gel. Requiring an introduction pump to take samples, the system would be relatively complex. Moreover, a complex column-format preparative electrophoresis device with complicated means of operation, expensive instruments and excessive dilution of elute is described (24). In ihe present investigation, a new native preparative electrophoresis system involving a modified device with a sample-receiving chamber and an assay method using Coomassie G-250 to trace the collection of the protein of interest were developed. The new system is extremely simple and promising for application in laboratories; it is very simple for many standard commercial apparatuses with cooling systems, e.g., a Hoefer SE 260 would be modified by adding and fixing suitable dimensions for the sample-receiving chamber. We believe that it can be conveniently set up in many locations without large expense. Isoelectric electrophoresis processes use an ampholyte mixture to establish a pH gradient. Ampholytes and other buffer modifiers are expensive, so the new system, which involves the use of a common preparative polyacrylamide gel, will offer a satisfactory resolution for this problem and decrease processing costs. Moreover, the major problem associated with the preparative polyacrylamide gel electrophoresis (Prep−PAGE) technique was inefficiency of heat dissipation, particularly overheating of the gel column, which results in both loss of stability and uniformity within the separating media. A condensing chamber (Figure 1) under the cathode electrophoresis buffer tank is applied to prevent heat dissipation during the electrophoresis separation. Additionally, the new system also adopts a sample-receiving chamber that includes semi-permeable membranes through which the buffer ion enters freely from all points and the macromolecular mass can be blocked, which results in a ready elution of separated components being collected and condensed. In our device, electrophoresis is briefly interrupted to withdraw the eluted fraction with a syringe. The collection volume is then replenished with buffer and the electrophoresis can continue.

Although vertical preparative SDS−PAGE is another cheap and reproducible method for quantifying, comparing and purifying proteins (34), this method is tedious and cannot be used for analysis of intact protein complexes in which biological activity needs to be retained for functional testing (27). Native electrophoresis would effectively prevent the protein of interest from denaturation and degradation. As a result, development of the preparative native electrophoresis would be more practically important.

For the preparative electrophoresis system, the most difficult problem is how to monitor the protein of interest and determine the optimal time for collecting it. In previous devices (35, 36), many attempts were made, involving double-material beam ultraviolet-visible spectrophotometer (37) and fluorescence intensity (24). Particularly during fluorescence intensity detection, the entire apparatus must be operated under a dark readbox to avoid ambient light. As a result, the method is complex and very tedious. In our new native preparative electrophoresis system, the convenient assay method is very simple, with Coomassie G-250 tracing the collection of the protein of interest.

Coomassie dye may nonspecifically combine together with proteins, which is very important for protein purifying using Coomassie dye to trace protein migration during gel electrophoresis (38). However, would the protein combining with dye change the migration of the testing protein? An experiment was designed to evaluate the migration velocity of the identical sample mixed separately with and without Coomassie G-250 loading buffer. The result is shown in Figure 5. A similar migration velocity electrophoresis of samples mixed with different buffers was found. Furthermore, the same result was found both a high molecular protein (asparaginase from fermentation broth produced in *E. coli*, Mr = 132,000) and low molecular protein (Synthetic Tα1, Mr = 3,108). The results further confirmed that the testing protein combining Coomassie G-250 will not change the electrophoresis swimming trail. Moreover, during the native electrophoresis, the color band of the Coomassie G-250 combining protein is vividly discerned and does not fade out as does Bromphenol Blue. On all accounts, the results demonstrate that the Coomassie G-250 combining

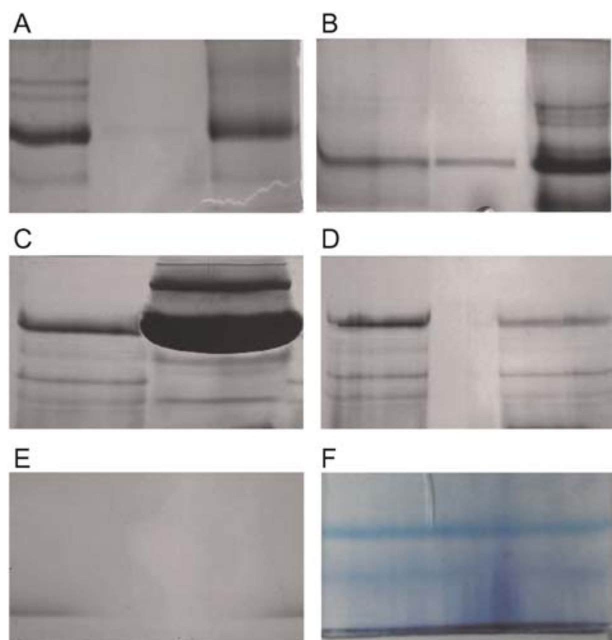

Figure 5. Electrophoresis mobility comparison among proteins of different molecular mass mixed with or without coomasses G-250 loading buffer. The samples mix separately with the aforementioned buffer A and B and simultaneously were loaded into native gel. The gels were stained and destained as the aforementioned method. In figure A and B, the left and right lane separately represents the protein of interest mixed with buffer A and B. The figure A and B represent profile of sample electrophoresis mobility at the beginning and the end of the native preparative gel. Figure C and D represent the fermentation sample mixed with buffer A and the standard protein (Asparaginase, 132 Kd) mixed with buffer B. Figure E represents synthetic thymosin mixed with buffer A and B. Figure F represents the profile of the tracing band in the native preparative gel during the electrophoresis.

protein can trace electrophoresis swimming of the protein of interest (Figure 5F). A combination of G 250 (only Mr = 854) to the testing protein has no or very little effect on the size of the protein, however, it can change the charge character of testing protein, which may be a possible explanation.

With different batches of samples in duplicate tests, a preferable reproducibility was observed and the yield of interest sample can reach 71.6–92%, which is close to electrophoretically pure. As shown in Figure 4, with different proteins from high relative molecular mass to low relative molecular mass, the method for the direct visualization of Coomassie Blue-stained polypeptide bands during native Prep-PAGE displayed similar and fine resolution. In addition, in this device, the electrophoresis sample capacity was 1.2–1.4 mg. The sample preparation process does not include steps for heating and adding denaturing agent–SDS. The collecting of the protein of interest is finished in one hour, and the harvested protein only contains the electrophoresis buffer ingredient, which can be readily settled by desalination with dialysis. Of course, there is a disadvantage in our instrumental design: that the device cannot run unattended during operation; that only a syringe is used to withdraw protein from the open sample receiving chamber; and for more complex samples with manifold proteins or protein complexes in native gels, the employment of this method may possibly be laborious. Moreover, compared with Lim's device tracing with 125I with one lane of the gel (33), our device would inevitably lose some sample, depending on tracing gel with Coomassie, and another approach including small slots of the comb would have to be employed.

Fusion protein is classically purified by a combination of several techniques, such as ammonium sulphate precipitation, affinity chromatography (Blue-Sepharose 6 F.F), hydrophobic chromatography and ion-exchange chromatography (13, 31, 32). Purification procedures are often long and complex. The native preparative electrophoresis system involves a modified device with a sample-receiving chamber and assay method with Coomassie G-250 tracing the collection of the protein of interest, which makes the collection of the protein of interest separating from gel more convenient and the protein of interest is concentrated without its activity affected. Therefore, this system greatly simplifies the separation process. This system will offer a new, rapid and simple method for the purification of the protein of interest.

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