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Purification of A Recombinant Thrombin-like Enzyme, Gloshedobin by Egg Yolk Antibody-Coupled Adsorbents

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Abstract: The gloshedobin, a snake venom thrombin-like enzyme was biosynthesized in the soluble form and purified by egg yolk antibody-coupled adsorbents from E. coli. As His-tag is not favored from the point of view of high-level and soluble expression, we herein constructed a recombinant gloshedobin without His-tag and developed a novel egg yolk antibody (IgY)-immunoaffinity chromatography for its purification in a higher activity yield. The purification process involving Octyl Sepharose FF, IgY-immunoaffinity chromatography and Source Q, yielded 454.7U mg ^{1} protein of interest and 34.8% activity recovery. The anti-gloshedobin IgY was obtained from eggs by injecting diluted gloshedobin into the breast muscle of laying hens and then purified by several steps including 3.5%, 8.5% and 12% polyethylene glycol-6000 precipitation and affinity chromatography using gloshedobin-coupled agarose gel. The obtained IgY was covalently linked to CNBr pre-activated agarose gel, Sepharose 4B FF, to yield immunoaffinity adsorbents. Both immunological and enzymatic activities of the purified enzyme were determined by western-blotting analysis and fibrinogen clotting assay, respectively.

Key words: *Escherichia coli*, Expression, Egg Yolk, Immunoaffinity Chromatography, Purification, Thrombin-Like Enzyme

INTRODUCTION

Thrombin-like enzymes belong to serine proteases and are characterized by their abilities to specifically hydrolyze fibrinogen to produce noncross-linked fibrins, which are more susceptible to the lytic action of plasmin than the thrombin-induced cross-linked clots [1]. Current studies showed thrombin-like enzymes involving ancrod from *Agkistrodon rhodostoma* [2] and defibrase from *Agkistrodon halys* and *Agkistrodon acutus* [3], caused patients only a modest increase in bleeding risk, showing they are of significant importance in the prevention and treatment of a wide range of thrombotic disorders [4, 5].

Various thrombin-like enzymes from different species of snake venom have been isolated and partially characterized, since Esnoff discovered the first thrombin-like enzyme from *Agkistrodon rhodostoma* in 1967 [6]. However, the amount of these enzymes purified from snake venom are not sufficient for both biochemical and molecular biologic properties studies, thus large-scale production of them by microorganisms seem to be an good alternative. So far several thrombin-like enzymes including batroxobin [7], mucrosobin [8], pallabin [9] and acutin [10] have been produced in *E.coli* as inclusion bodies. In most cases, a fused 6×his-tag was designed to facilitate purification process. The major problem with this strategy, however, is that this attached peptide tag may alter the folding of the protein and in turn change protein properties involving structural, physiological and pharmacological features [11]. Therefore, to develop equally efficient purification approach is of biotechnological importance. Among affinity chromatographies, immunoaffinity chromatography is in principle of great value in that the corresponding antibody as ligand is of the highest affinity and selectivity to a given protein, either the natural one or the recombinant one [12]. Compared with high-cost monoclonal antibodies, polyclonal antibodies like egg yolk antibody is of practical advantages to produce in large-scale.

Gloshedobin is a snake thrombin-like enzyme from the venom of *Gloydius shedaoensis*. We previously report its biosynthesis in *E. coli* and its purification by using a 6xhis-tag [13]. Since His-tag is not favored from the point of view of high-level and soluble expression, we herein constructed a recombinant gloshedobin without His-tag and developed a novel IgY immunoaffinity chromatography for its purification in a higher activity yield.

MATERIAL AND METHODS

Construction of Expression Vector: The open reading frame of the cDNA sequence encoding gloshedobin [14] (GenBank accessory AJ278786.1) was amplified by PCR using upstream primer 5'-GGGAATTCATCATTGGAGGTGATGAATG-3' and downstream primer 5'-GTCTCGAGTCATGGGGGGCAGGTTGCAT-3',

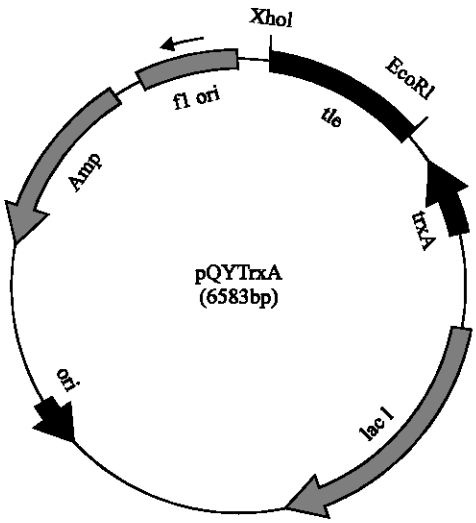

Fig.1: Schematic Diagram of pQYTrxA, an Expression Vector Derived from pET32a (+) Vector. "tle" Represents the Gene of Gloshedobin

respectively. The resulting PCR products included two restriction sites, *Eco*R I at its 5' terminal and *Xho* I at its 3' terminal. The PCR reaction was carried out for 30 cycles under the conditions involving denaturation at 94°C for 1 min, annealing at 55°C for 45 sec and elongation at 72°C for 40 sec. The PCR product was purified and ligated with linear pMD-18 simple T vector to result in pMDQY. After digestion with *Xho* I and *EcoR* I, the targeted gene released from pMDQY and ligated to pET-32a (+) vector (Novagen, USA) resulting in the expression plasmid pQYTrxA (Fig. 1).

Expression of Recombinant Gloshedobin: The expression plasmid pQYTrxA derived from plasmid pET-32a (+) (Novagen, USA) were used to transform *E. coli* strain BL21 (DE3) competent cells [15]. The recombinant *E. coli* cells were grown at 37°C in Luria broth containing 100 μg ml^{-1} ampicillin overnight, then inoculated into fresh medium and grown at 37°C until the cell dry weight reached 0.2 mg ml^{-1}. Expression of the recombinant enzyme was induced by addition of isopropyl -D-thiogalactopyranoside (IPTG) at final concentration of 1mM. After induction, the harvested cells were centrifuged at 10,000g for 10 min and resuspended in TE buffer (20 mM Tris-HCl, pH 7.9, 1 mM EDTA). Cells were lysed by ultrasonication and centrifuged at 12,000g for 20 min. The supernatant containing soluble recombinant gloshedobin was removed and stored at -20°C for the purification step. The inclusion bodies fraction was denatured in 20 mM Tris-HCl (pH 7.9), 5 mM imidazole, 8 M urea, 0.5 M NaCl at 4°C overnight. After centrifugation at 5,000g for 20 min, this supernatant was utilized as immunogen material.

Preparation of Inclusion Bodies as Immunogen: The prepared inclusion bodies sample above was loaded on Ni^{2+}- IDA Sepharose affinity column according to the manufacturer's instruction (Amersham Biosciences, Sweden), washed with 20 mM Tris-HCl (pH 7.9), 60 mM imidazole, 8 M urea, 0.5 M NaCl and eluted by 20 mM Tris-HCl (pH 7.9), 500 mM imidazole, 8 M urea, 0.5 M NaCl. The target fraction was loaded on Sephadex G-25 to change buffer to PBS (20 mM phosphate buffer saline, pH 7.4). Then approximately 500 μg μl^{-1} of the purified gloshedobin (IBs) mixed with an equal volume of complete Freund's adjuvant (Gibco, USA) used as immunogen. Laying hens were immunized by injecting the immunogen into the breast muscle following twice enhanced immunization using incomplete Freund's adjuvant performed with 2-week interval. One month later, the eggs were collected for further extraction.

Extraction of IgY from Egg Yolk: The extraction of IgY from egg yolk was undertaken by two-step precipitation with PEG-6000 according to Polson's methods [16]. In brief, 160 ml egg yolk was diluted to 800 ml with 0.1 M phosphate, pH 7.6. PEG-6000 was added to give a final concentration of 3.5% (dilution, w v^{1}). Then the mixture was incubated at room temperature for 10 min. After centrifuged at 5,000g for 30 min, the mixture was separated into three phases: a semi-solid lipid precipitate, a clear water-soluble protein fraction and a yellow fatty layer floating on the surface. The soluble protein fraction and the yellow fatty layer were decanted off through filtration. Then the addition of 8.5% (dilution, w v^{1}) pulverized PEG-6000 to the soluble protein fraction, followed by the incubation at room temperature for 10 min and centrifugation at 5,000g for 30 min, resulted in the IgY precipitates. The sediment was dissolved in 400 ml 0.1 M phosphate buffer, pH 7.6. And the IgY was precipitated again with 12% (dilution, w v^{1}) PEG-6000. The pellets were obtained by subjecting to a centrifugal force 10,000g for 25 min and dissolved in 40 ml 0.1 M phosphate buffer, pH 7.6, followed cooling to 0°C. 40 ml 50% ethanol pre-cooled at -20°C (dilution 1:1, v/v) was added, stirred and centrifuged at 10,000g for 20 min at 4°C. This cryo-ethanol step removed all residual PEG components in the precipitate. After the precipitation, the IgY sediment was dissolve in 8 ml of 20 mM Tris-HCl (pH 7.5).

Affinity Separation of IgY Antibodies: Two gram CNBr-activated Sepharose FF gel (Amersham Biosciences, Sweden) was washed for three times with 400 ml 1 mM HCl and then mixed with 40 mg purified gloshedobin in 20 ml coupling buffer, 0.5 M NaCl/0.1 M $NaHCO_3$, pH 8.3. After incubation for 1 hr at 25°C, the Sepharose FF gel was stirred slightly and washed with 5-fold volume of coupling buffer, 0.5 M NaCl/0.1 M $NaHCO_3$, pH 8.3. Resuspend the gel in 0.1 M Tris-HCl buffer, pH 8.0, for 2 hr to block the residual active site of the gel. The resulting gel was then washed cross-repeatedly by 5-fold volume of coupling buffer, 0.5 M NaCl/0.1 M $NaHCO_3$, pH 8.3 and buffer, 0.5 M

NaCl/0.1 M acetate buffer, pH 4.0. Finally, gloshedobin-Sepharose FF gel was packed into a XK16/20 column (Amersham Biosciences, Sweden) and equilibrated with at least 5-fold-column volume of 20 mM Tris-HCl buffer, pH 7.5. The isolated IgY fractions were loaded into the column at a flow rate of 1 ml min ^{1}. The column was washed with 5-fold-column volume buffer, 20 mM Tris-HCl, pH 7.5 and bounded anti-gloshedobin IgY antibodies were eluted with 0.2 M glycine-HCl (pH 2.8). For immediate neutralization, the eluted fractions (3 ml) were poured into 5 ml-tubes containing 0.18 ml of 1 M Tris-HCl buffer (pH 8.0).

Preparation of Anti-gloshedobin IgY Immunoaffinity Chromatography: Coupling reaction was performed according to the methods described in section, '*Affinity purification of IgY antibodies*'. 40 mg anti-gloshedobin IgY was coupled to 7 ml of CNBr-activated Sepharose Fast Flow gel (Amersham Biosciences, Sweden). More than 93% IgY was coupled to the gel by measuring the amount of uncoupled proteins. Thus, anti-gloshedobin IgY immuno-affinity adsorbents were prepared.

Purification of the Recombinant Gloshedobin: Cells were lysed by ultrasonication and then centrifuged at 10,000g for 15 min. The precipitates were discarded and the supernatant containing soluble recombinant gloshedobin was kept for the followed purification steps including hydrophobic chromatography on Octyl Sepharose FF column, immuno-affinity chromatography on IgY Sepharose 4B FF and ion-exchange chromatography on Source Q. The operation procedure of purification strategies was described as follows: 1) Hydrophobic chromatography. Solid ammonium sulfate was added into the supernatant till the final concentration was up to 1.2 M. The supernatant were adjusted to pH 7.5 and then was pumped into Octyl Sepharose FF column, equilibrated with buffer, 20 mM Tris-HCl containing 1.2 M ammonium sulfate, pH 7.5. The target protein was eluted by a linear gradient of ammonium sulfate from 1.2 M to 0 M. Remove the salt from the collections by using Sephadex G-25; 2) Immunoaffinity chromatography. The effluent was loaded to anti- gloshedobin-IgY immunoaffinity chromatography column equilibrated with buffer, 20 mM Tris-HCl, pH 7.5. The protein of interest was eluted with a linear gradient of 0-1.0 M NaCl in 20 mM Tris-HCl, pH 7.5. The fractions containing the target protein were collected; 3) Ion-exchange chromatography. The active collection obtained from immunoaffinity chromatography was applied onto Source Q column. The protein of interest was eluted with a linear gradient of 0-0.5 M NaCl in 20 mM Bis-Tris, pH 6.5. The purification efficiency of each chromatographic step was evaluated by SDS-PAGE assay.

Protein Concentration Estimation: The protein concentration was estimated according to Bradford's method [17] using bovine serum albumin (BSA) as standard.

SDS- polyacyl Amide Gel Electrophoresis: Sodium dodecyl sulfate polyacyl amide gel electrophoresis using Tris-Tricine buffer was performed according to the method of Laemmli [18].

Western Blotting Assay: Proteins on SDS-PAGE gel were transferred onto a PVDF membrane at 0.65 mA cm ^{2} for 3 hr. This membrane was then blocked in 2% fat-free milk at room temperature for 1 hr and incubated in the same buffer containing horse anti-snake venom serum from *Agkistrodon halys* (dilution 1:500, v/v) for 1 hr. Washed with buffer (150 mM NaCl, 5% Triton, 20 mM Tris-HCl, pH 8.0), the membrane was transferred into 2% fat-free milk containing rabbit anti-horse IgG conjugated to horseradish peroxidase (dilution 1:1000, v/v) for 1 hr. Washed the membrane with buffer (150 mM NaCl, 5% Triton, 20 mM Tris-HCl, pH 8.0). Finally antigen-antibody binding was detected using 3, 3 -diaminobenzidine (DAB) and hydrogen peroxide kit.

Determination of Antibody: The binding activity of IgY with gloshedobin was determined by sandwich ELISA method monitored at 492 nm by Sunrise automatic ELISA-meter (Tecan, Austria). Briefly, 100 μl 20 μg ml ^{1} recombinant gloshedobin solution in 20 mM Tris-HCl, pH 7.9, was added to each well of a 96-well plate and incubated at 4°C overnight. Then the plate was washed with TBS-T buffer, (TBS-0.05% Tween-20) for three times. 100 μl of 1:500 diluted yolk was added to each gloshedobin-coated well and incubated at 37°C for 2hr. Plate was washed again with TBS-T buffer for three times. Then 100 μl of 1:250 diluted alkali phosphatase-conjugated rabbit anti-chicken IgG (Sigma, USA) was added to each well and incubated at 37°C for 2 hr. Each well was again washed with TBS-Tween for three times, then was added 100 μl freshly prepared substrate solution (10 mg ml^{-1} citrate sodium, 0.4 mg ml^{-1} OPD, 0.1 M phosphate disodium, 1.5 μl ml^{-1} 30% H_2O_2 (dilution, v/v). 200 μL of 2 M H_2SO_4 was applied to each well to stop the reaction after a 30 min incubation at 25°C.

Amidolytic Activity Assay: Amidolytic activity was assayed using chromogenic substrate N- -p-tosyl-Gly-Pro-Arg-p-nitroanilide (Sigma, USA). One unit of amidolytic activity was defined as the amount of enzyme necessary to hydrolyze 1.0 μmol min ^{1} of substrate [19]. Ancrod, commercially available thrombin-like enzyme from sigma, was used as standard.

RESULTS AND DISCUSSION

Preparation of Immunogen: Since the large amount of the inclusion bodies was available, we used recombinant gloshedobin in the form of inclusion bodies as antigen for the production of IgY antibodies.

Table 1: Purification Efficiency of Anti-Gloshedobin IgY Purified by Gloshedobin-Immunoaffinity Column; Specific Activity=ELISA Value mg ^{1} of Total Proteins

Steps	Specific activity	Purification fold
Precipitation of 8.5% PEG-6000	0.3	1.0
Precipitation of 12% PEG-6000	0.8	2.6
Gloshedobin-immunoaffinity chromatography	60.0	200.0

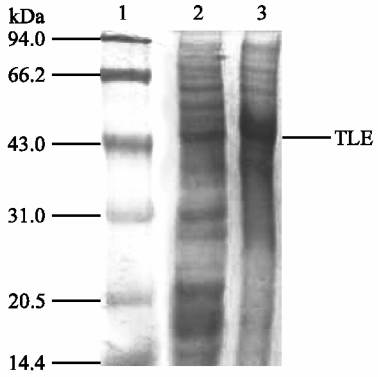

Fig. 2: 10% SDS-PAGE Analysis of Recombinant Gloshedobin Produced, Lane 1, LMW Protein Maker; Lane 2, Total Soluble Proteins; Lane 3, Total Insoluble Proteins; TLE Represents the Thrombin-Like Enzyme, Gloshedobin

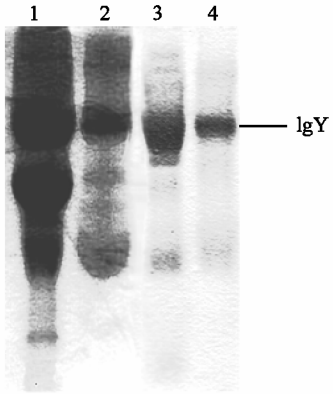

Fig. 3: SDS-PAGE Analysis of the Purification Procedure of Specific IgY, Lane 1, Crude IgY; Lane 2, The Isolated IgY by 3.5% and 8.5% PEG; Lane 3, The Partially Purified IgY by 12% PEG; Lane 4, The IgY Purified by Using TLE-Sepharose Immunoaffinity Column

To facilitate the purification, we applied the expression strain carrying an expression vector with 6xHis-tag because the bioactivity of the immunogen was not cared in case of preparation of immunogen. Induced by IPTG at 40°C for 8 hours, approximately 70% recombinant gloshedobin of total cellular proteins was inclusion bodies (Fig. 2). The purification of inclusion bodies was firstly performed by Ni^{2+} immobilized chelating chromatography column under the denature condition (data not shown) and then by PEG 6000 (polyethylene

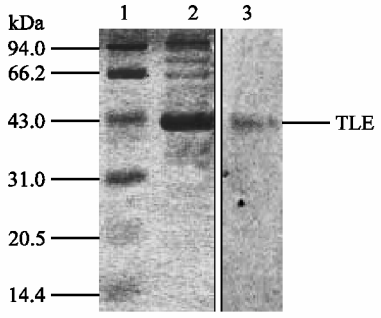

Fig. 4: Lane 1, LMW Protein Maker; Lane 2, SDS-PAGE Analysis of the Purified Recombinant Gloshedobin; Lane 3, Anti–Venom Serum Western-Blotting Analysis of the Purified Recombinant Gloshedobin

glycol 6000) precipitation at different concentrations (Fig. 3 and lane 2-3). Further purification was performed on gloshedobin-affinity adsorbents and the highly purified IgY was eluted by 0.1 M Gly-HCl, pH 2.8 (Fig. 3 and lane 4). SDS-PAGE showed the purity of the resulting IgY (Fig. 3 and lane 4) and ELISA assay showed the specific activity of IgY increased evidently from 0.3 to 60 after the purification procedure (Table.1).

Purification of the Recombinant Gloshedobin: Compared with mammalian animals, hens are excellent alternatives to generate polyclonal antibodies in that: 1) relatively inexpensive chicken housing, 2) non-stressful to hens for egg collection and 3) fast and simple isolation of IgY antibodies. In addition, the fact that yolk immunoglobulins do not activate mammalian Fc receptors, which could mediate inflammatory response in the gastrointestinal tract, makes IgY antibodies very attractive for peroral immunotheraphy [20]. In this work, we used eggs yolk polyclonal anti-gloshedobin antibodies as ligands for the purification of the recombinant gloshedobin. The recombinant enzyme in the supernatant was separated and purified by the three steps sequentially involving` Octyl Sepharose FF, anti-*gloshedobin* IgY immunoaffinity chromatography and Source 15Q. The protein recovery efficiencies of the corresponding purification steps were 87.1%, 42.1% and 11.1%, respectively. In addition, the amidolytic activity recovery efficiencies were 71%, 69.2% and 34.8% (Table.2). The specific amidolytic activity of purified product by each step is

Table 2: Purification of the Recombinant Gloshedobin from E.coli

Purification	Protein			Amidolytic activity			
Steps	Total (mg)	Gloshedobin (mg)	Recovery (%)	Total (U)	Specific activity (U mg ^{1})	Recovery (%)	Purification(fold)
Crude	80.5	6.84	100.0	1240	15.4	100.0	-
HIC	33.3	5.96	87.1	880	26.4	71.0	1.7
IAC	8.96	2.88	42.1	858	95.8	69.2	6.2
Source Q	0.95	0.76	11.1	432	454.7	34.8	29.5

26.4, 95.8 and 454.7U mg ^{1} (Table.2) and the purified recombinant gloshedobin gave one band on SDS-PAGE (Fig. 4, lane 2).

Bioactivity Analysis: The western blotting assay (Fig. 4, lane 3) indicated that the anti-venom serum recognized the purified recombinant gloshedobin. As the recombinant gloshedobin without cleaving fusion partner exhibited obvious fibrinogen-clotting activity, therefore we concluded that thioredoxin had no negative effect on bioactivity of gloshedobin (data not show).

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