Purification of *Escherichia coli* L-asparaginase Mutants by a Native Polyacrylamide Gel Electrophoresis

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Abstract

The antigenicity of L-asaparaginase (L-ASP) has been problematic for the treatment of leukemia for many years. In order to establish a relationship between the antigenic epitope of L-asparaginase and its antigenicity, several L-asparaginase mutants (mL-ASPs) are constructed and expressed. To effectively purify these enzyme mutants for further investigation, a native preparative polyacrylamide gel electrophoresis is developed. The simplicity and reproducibility of this approach permits the purification of different mutants from the crude enzyme extracts, with a sufficient activity to perform immunological and biological studies. Furthermore, the newly developed method is efficient and cost-effective compared with other methods, such as column chromatography and affinity chromatography. As a result, the enzyme mutants with specific activity of 300~400 U/mg are obtained by the single-step purification with a high degree of purity.

Introduction

L-asparaginase (L-ASP, EC.3.5.1.1) has been used in the treatment of acute lymphoblastic leukemia, acute mielomonocitie leukemia, lymphosarcome, and melanosarcoma (1–2) based on the conversion of L-asparagine (L-Asn) to L-aspartate and ammonia. The mechanism of L-ASP cytotoxicity is thought to be the deficient or absent of L-Asn synthetase activity in leukemia cells, indicating that protein synthesis and cell proliferation are closely related to the extracellular level of asparagine. Therefore, a supply of L-ASP can effectively reduce blood L-Asn concentration to selectively inhibit malignant cell growth for the treatment of leukemia (3). Unfortunately, serious side effects, especially immunological reactions, often occur in the L-ASP therapy, which limits its therapeutic utility in clinics. Because the antigenicity of L-ASP is likely responsible for the side effects in clinical uses, different L-ASP mutants (mL-ASPs) were constructed and expressed in our lab (4) in order to elucidate the mechanism of antigenicity of L-ASP and the roles of specific residues. For this reason, a large amount of pure and active enzymes is required to study the effects of different mutants. Because protein purification is typically tedious and labor-intensive, an efficient, simple, and economical method to overcome these obstacles is obviously preferable and advantageous. Polyacrilamide gel electrophoresis (PAGE) has been commonly used in protein analysis due to its high resolution, but the relative higher expenses and denaturing conditions of preparative gel electrophoresis considerably limit its practical application as a method for protein purification. In the present study, we have developed a native preparative (NP) gel electrophoresis system based on double electrophoresis and elution through a membrane to obtain pure proteins retaining respective activity and the application of this new NP-PGAE for the preparation of mL-ASPs.

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Experimental

Materials

E. coli pKA/CPU210009-mL-ASP I–*E. coli* pKA/CPU210009-mL-ASP IV were constructed and expressed for L-ASP mutants as described previously (4). Mid-range protein markers were obtained from Promega (Shanghai, China). Acrylamide and N,N'-methylene bisacrylamide were from BBI (Ontario, Canada). Glycine and Tris were purchased from Amresco (Solon, OH). All other chemicals and reagents were of analytical grade.

Instrumentation

The circulating cooler was from Bio-Rad (Hercules, CA). DYCZ-23A electrophoresis equipment used was purchased from Beijing LiuYi instrument factory (Beijing, China). The improved gel-carrying plate and spacers were redesigned by our lab and produced by Nanjing Chishun Sci. & Tech. Developing Co., Ltd (Jiangsu, China).

Procedure

Preparation of the crude enzyme extract

Bacterial cells were suspended in a five-fold volume (w/v) of lysis buffer, including 45% (w/v) sucrose, 10mM/L EDTA, and

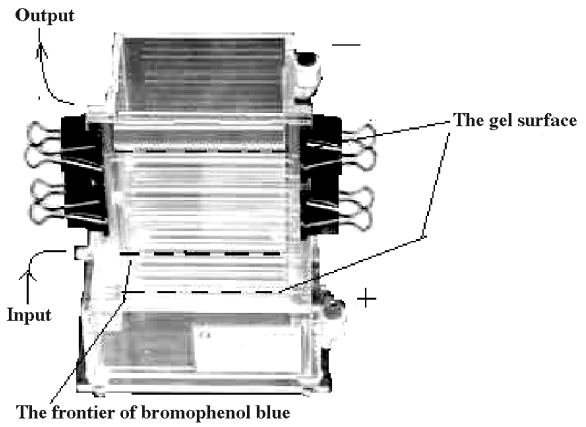

Figure 1. The downward stage of the native PAGE.

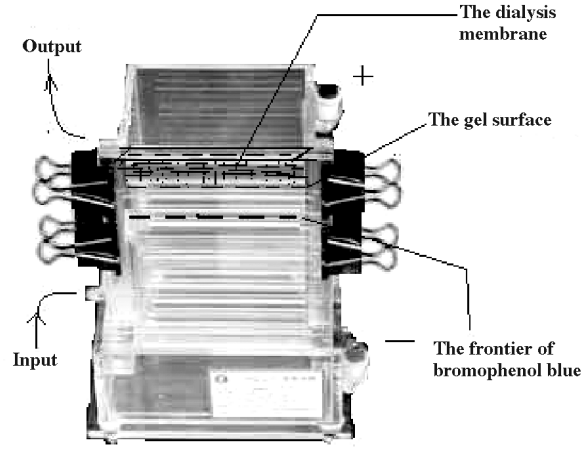

Figure 2. The upward stage of the native PAGE.

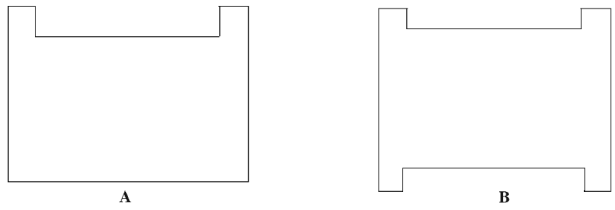

Figure 3. The inward gel-carrying plate. The normal inward gel-carrying plate (A); the inward gel-carrying plate with two notches (B).

200 mg/L lysozyme, pH7.5. After the suspension was incubated for 70 min at 32°C with continuous stirring, the same volume of 2% MnCl2 solution (w/v) was added. The mixture was first kept at 4°C with continuous stirring for 60 min to sedimentate the nucleic acid, and then centrifuged at 4°C and 8000 r/min. Solid ammonium sulfate was added to the resulting solution, and the precipitate formed between 65% and 90% saturations was collected by centrifugation. The precipitate was dissolved in the 10mM sodium phosphate buffer pH6.4 and dialyzed against the same buffer. The final solution obtained was called and used as crude enzyme extract.

Purification of mL-ASPs

The inward gel-carrying plate was redesigned as the one with two notches. The spacers between the two gel-carrying plates were increased from the usual 1.5 mm to 3 mm. The tabular gel was made of 20 mL of 7.5% polyacrylamide separating gel and 3 mL of 3.5% polyacrylamide stacking gel. One milliliter sample (containing approximately 2~3 mg proteins) was loaded onto the gel containing 100 µL of bromophenol blue to monitor the electrophoretic front. The circulating cooler was connected to the electrophoresis equipment when the electrophoresis started with a *Tris*–glycine buffer (25mM *Tris*, 250mM glycine, pH 8.3). A constant current of 10 mA was applied when the electrophoretic front was in the polyacrylamide stacking gel, and 20 mA in the polyacrylamide separating gel, which was designated downward electrophoresis, Figure 1. After 5 h, when the bromophenol blue band ran into the lower tank buffer, the electrophoresis was stopped and the gel-carrying plates were reversed. Meanwhile, the dialysis membrane was placed between the notch of the upper tank and the inward gel-carrying plate to make a collecting chamber that was formed by the outward gel-carrying plate and the dialysis membrane. The chamber was filled with 2 mL of fresh electrophoretic buffer. The upper and the lower tank buffers were replaced with fresh electrophoretic buffer, and cathode and anode electrodes were exchanged. A constant current of 20 mA was applied in the upward electrophoresis as shown in Figure 2. After 3.5 h, the fraction in the collecting chamber was discarded. The collecting chamber was washed three times with fresh electrophoretic buffer, and then filled with 2 mL of fresh electrophoretic buffer. Finally, mL-ASPs were eluted from the gel during the following 3 h.

Assay for mL-ASPs activity

The collected fractions containing mL-ASP were mixed with

Table I. Purification of mL-ASPs from *E. coli* Crude Enzyme Extract

	Total protein (mg)				Total activity (U)				Specific activity (U/mg)				Folds of purification			
	M_1*	M_2*	M_3*	M_4*	M_1	M_2	M_3	M_4	M_1	M_2	M_3	M_4	M_1	M_2	M_3	M_4
Crude enzyme extracts	2.38	2.86	2.94	2.42	389.86	595.39	617.52	541.98	163.81	208.18	210.04	223.96	1	1	1	1
Native PAGE	0.21	0.26	0.23	0.25	73.34	99.55	90.25	101.26	349.24	382.9	392.39	405.03	2.1	1.84	1.87	1.81

* M_1, M_2, M_3, and M_4 represent mL-ASPI, mL-ASPII, mL-ASPIII, and mL-ASPIV, respectively.

2 mL of 0.1 M borate buffer, pH8.4, containing 20mM L-Asn, and the reaction mixture was incubated for 15 min at 37°C. The reaction was stopped with the addition of 1 mL of 50% trichloroacetic acid. The amount of NH4+ was determined by the Nassler method (5). mL-ASPs activity was expressed in units (U). One unit was defined as the release of 1mM of ammonia from the substrate per min.

Assay for protein concentration

Protein concentration was measured by the modified Lowry method (6), with BSA as a standard.

Determination of the purity of mL-ASPs by SDS-PAGE

The purity of the purified mL-ASPs was analyzed by SDS-PAGE (12% gels), respectively. SDS-PAGE was carried out under reducing conditions. The gels were stained for protein with Coomaisie Blue R250.

Results

Method development

The development of this native preparative PAGE involved: (*i*) a redesigned inward gel-carrying plate with two notches as shown in Figure 3, which is convenient for two-step electrophoresis (downward and upward); (*ii*) running reversed electrophoresis by rearranging the gel-carrying plates after the bromophenol blue band runs into the lower tank buffer; (*iii*) making a collecting chamber between the outward gel-carrying plate and the dialysis membrane that was used to isolate the upper tank buffer; and (*iv*) adding a circulating cooler to control the running buffer at a desired temperature. Therefore, with the combination stated previously, specific proteins can be effectively separated and recovered with the original activity.

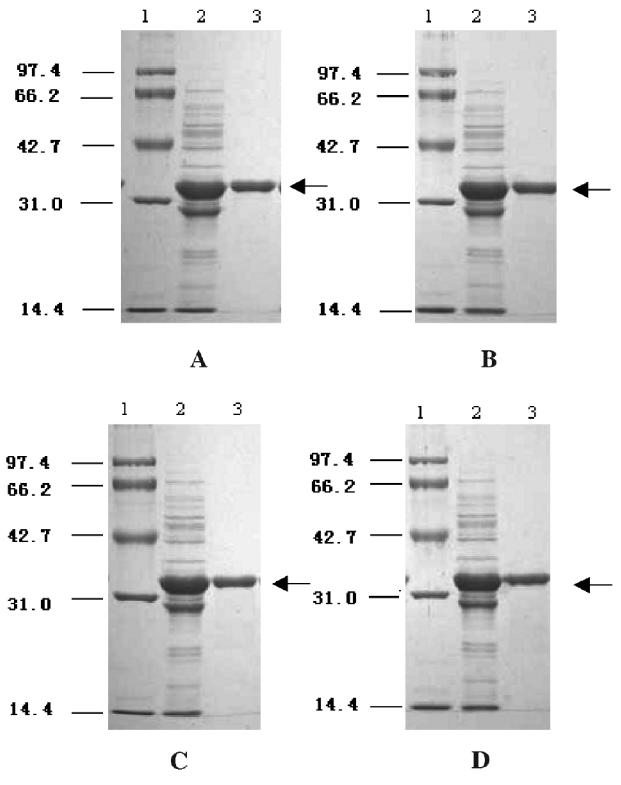

Figure 4. SDS-PAGE (12% gel) with the purified mL-ASPs. mL-ASPI (A); mL-ASPII (B); mL-ASPIII (C); mL-ASPIV (D). 1: Molecular size markers; 2: mL-ASPs from *E. coli* crude enzyme extracts; 3: Purified mL-ASPs. The gels were stained with Coomaisie Brilliant Blue R250. Molecular masses in kD are given on the left. The arrows show the positions of mL-ASPs.

Purification of mL-ASPs

mL-ASPs were chosen and utilized for protein purification with NP-PAGE. Purification results for different mL-ASPs are summarized in Table I. The specific activity of mL-ASPs in the crude enzyme extract was approximately 200 U/mg. After NP-PAGE purification, the specific activity of mL-ASPs was increased to 350 U/mg.

Determination of the purity of mL-ASPs by SDS-PAGE

The active fractions were analyzed by SDS-PAGE (12% gel). Each purified mL-ASP exhibited a single predominant band as shown in Figures 4A–D, indicating that the NP-PAGE is able to effectively purify active proteins with high purity.

Discussion and Conclusion

L-ASP was a tetramer composed of four identical subunits. Each subunit has a molecular mass of 33 .796 kD (7), and an isoelectric point (pI) of 4.85 (8). The residues we chose for mutagenesis were basic amino acids, including Lys, His, and Arg, which may play an important role in the antigen-antibody interaction (9). Mutagenesis of these residues may result in the change of the surface charge of these mutants [mL-ASP I (R195A, K196A, H197A); mL-ASP II (K196A, H197A); mL-ASP III (R195A, K196A); mL-ASP IV (R195A, H197A)], which showed different mobility on the native PAGE (Figure 5). Because the mutations

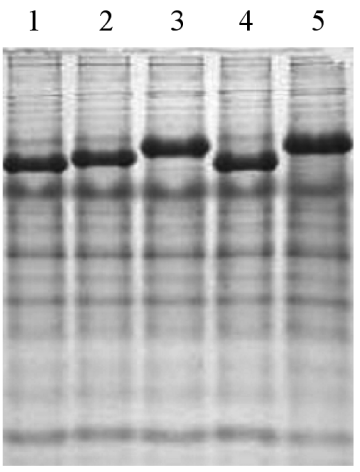

Figure 5. Native PAGE (10% gel) with mL-ASPs from bacterial cells. mL-ASPI (lane 1); mL-ASPII (lane 2); mL-ASPIII (lane 3); mL-ASPIV (lane 4); wild type L-ASP (lane 5). The gels were stained with Coomaisie Brilliant Blue R250.

resulted in changes in pI and other physical and chemical properties of the proteins, it would be very difficult to use a general chromatographic method to efficiently purify these proteins. On the other hand, we found that NP-PAGE with two-step electrophoresis and elution through a membrane for protein recovery provided an efficient and versatile method for the preparation of pure protein samples. In the case of mL-ASPs, NP-PAGE has shown its great power for purification. Just after one step purification, the specific activity was increased from 200 U/mg to 350 U/mg without any contaminating proteins judged by analytical SDS-PAGE. During the electrophoresis, most of the contaminating proteins were migrating out from the gel after 3.5 h upward direction, and other contaminating proteins were effectively separated by reverse direction with fresh buffer. We noted that a change of buffer between downward and upward directions was critical for the protein purification. Although the mechanism of protein separation is unclear, we speculate that the physical and chemical properties for a particular protein are quite different, especially surface charge of the protein, under different buffer conditions. Such interesting and unusual behavior has prompted us to further investigations. In summary, we have developed a simple, reliable, and efficient NP-PAGE method to rapidly purify protein mutants that retain the original activity. This approach will ultimately be very useful in many areas of biochemical studies.

Acknowledgments

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