An efficient and reliable electroelution method from SDS-PAGE: Identification of a 31 kDa protein in the postsynaptic density fraction as adenine nucleotide translocator 1

Jae-Seob Jung[1], Sun-Jung Cho[1], Seung Chul Shin[2], Ingnyol Jin[1], Yong Wook Jung[2], Bok Hyun Ko[2] and Il Soo Moon[2]*

*Department of Microbiology, College of Natural Sciences, Kyungpook National University, Taegu 702-701, Korea
Department of Anatomy and Research Institute for Natural Science of Dongguk University, College of Medicine, Dongguk University, Kyongju 780-714, Korea*

Abstract

The molecular composition of the postsynaptic density (PSD) is largely unknown. In this report, an electroelution protocol was demonstrated to be used for efficient isolation of PSD proteins with diverse molecular sizes. Using this protocol, a 31 kDa protein in the 1% n-octyl glucoside-insoluble PSD fraction (termed as PSD31) was purified from SDS-gels, and internal peptides were determined for amino acid sequences. The amino acid sequences of the PSD31 were highly homologous with the adenine nucleotide translocator 1 (ANT1). The association of ANT1 with PSD suggests presence of a mechanism in synapses for releasing adenosine nucleotides into the extracellular space.

Key words – ANT1, electroelution, PSD, protein sequencing, SDS-PAGE

Introduction

The postsynaptic density (PSD) is a protein-rich cytoskeletal specialization that is tightly juxtaposed to the postsynaptic membrane. In addition to cytoskeletal proteins, many proteins of diverse functions are localized to the PSD, including neurotransmitter receptors, protein kinases, transcription factors, a putative cell adhesion molecule, scaffold proteins, and other regulatory proteins [8,9,18]. The PSD is now viewed as a dynamic structure involved in the synaptic transmission and regulation of synaptic plasticity by controlling synaptic membrane adhesion, receptor clustering, and regulation of receptor function [8,9,18]. However, our knowledge on the molecular composition of the PSD is still very limited

and most of the PSD proteins await identification.

A most straightforward method for identification of a protein is to determine its amino acid sequence. Unfortunately, most protein purification methods are inapplicable for PSD proteins mainly because of their highly hydrophobic nature. Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) is a powerful technique for the separation of complex protein mixtures and can be applied for purification of PSD proteins on both analytical and preparative scales. However, when a very complex protein mixture such as PSD is separated in SDS-gels, it is generally impossible to obtain an enough amount of target proteins in a single run, necessitating extraction and concentration of the proteins. Among several methods for the extraction of proteins from gels, electroelution can be conveniently adapted for extraction of multiple samples in large scales, and has been successfully applied for sequencing

*To whom all correspondence should be addressed
Tel : +82-54-770-2414, Fax : +82-54-770-2447
E-mail : moonis@dongguk.ac.kr

PSD proteins such as N-methyl-D-aspartate receptor subunit 2B [15], a putative cell adhesion molecule densin-180 [1], a synaptic Ras-GTPase activating protein p135 SynGAP [3], citron [23], glyceraldehyde-3-phosphate dehydrogenase [17], and a voltage-dependent anion channel 1 [16].

In the present study, it is shown that this electroelution protocol can be used for elution of PSD proteins with diverse molecular sizes, and that PSD31, sequenced using this protocol, is identified as adenine nucleotide translocator 1.

Materials and Methods

Isolation and fractionation of the PSD fraction: The One-Triton PSD fraction was prepared from adult rat (Sprague-Dawley) forebrains by washing the synaptosomes with a final 0.5% Triton X-100 as described by Moon *et al.* [15]. The One-Triton PSD fraction was treated with 1% n-octyl glucoside (OG) at 4°C for 30 min with stirring and the soluble (OG-S) and insoluble (OG-P) fractions were separated by centrifugation at 200,000 x g for 30 min at 4°C.

SDS-PAGE and electroelution: Electroelution of proteins after SDS-PAGE was essentially described by Moon *et al.* [15]. In brief, the OG-P fraction was electrophoresed in standard SDS-gels [11], using 10 mm-wide and 1.5 mm-thick loading wells instead of a single long preparative well. Proteins were stained for 30 min with 0.2% (w/v) Coomassie R-250 in a buffer containing 0.1% SDS and 10 mM Tris-HCl (pH 8.0) without fixatives. The gels were destained 3 times (each for 10 min) in the same solution without Coomassie dye. Gels were placed on a florescent light box and each protein bands were cut out with razor blades and chopped into small pieces (3-4 mm). Proteins were electroeluted in an Elutrap (Schleicher and Schuell) for 5-6 hr at 250 V using a buffer containing 25 mM N-ethylmorpholine (pH 8.5) and 0.01% SDS.

Sequencing PSD54 and PSD31: The PSD31 (300 μg) was

digested with trypsin (substrate:enzyme ratio, 1:1) in gel and the tryptic peptides were purified on a C_{18} reverse phase high pressure liquid chromatography column (RP-HPLC) as described by Moon *et al.* [16]. The amino acid sequences were determined by automated Edman degradation at the Biomolecule Research Group of Korea Basic Science Institute, Taejon, Korea.

Results

Efficient electroelution of multiple PSD proteins with various molecular sizes: In order to test if the electroelution protocol is applicable to PSD proteins of various molecular sizes, the 1% OG-insoluble One-Triton PSD proteins were fractionated in 6% SDS-gels, stained with Coomassie dye in a buffer without fixatives, and protein bands with diverse molecular sizes were
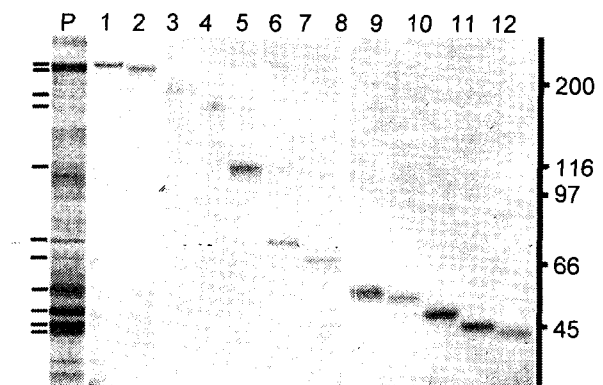

Fig. 1. Efficient elution of PSD proteins from SDS-gels. Proteins in the n-octyl glucoside (OG)-pellet (OG-P) fraction were separated in 6% preparative SDS-polyacrylamide gels and each protein band was electroeluted as described in detail under Materials and Methods. Equal relative volumes of eluant, corresponding to sixty micrograms of the OG-P fraction (lane P) were electrophoresed in a 6% SDS-gel, stained with Coomassie dye and destained as usual. The positions of each eluted protein band (lanes 1-12) in the NOG-P fraction were marked with bars. Molecular sizes were shown at far right in kilodaltons (kDa). Previously identified proteins are as follows: fodrins (1, 2), NR2B/citron/densin (4), tubulin (8), α CaMKII (10), and actin (11).

subjected to electroelution as described in detail under Materials and Methods. The OG-P fraction and each eluted protein were electrophoresed in a 6% SDS-gel and stained with Coomassie dye. As shown in Fig. 1, each protein band with various molecular sizes could be efficiently eluted from the gel. Densitometric analyses of the Coomassie-stained gel indicated that the elution efficiency for most of the bands was 60-80%. Futhermore, each PSD protein that constitutes more than 1% of the total fraction could be unambiguously identified and eluted without contamination with adjacent protein bands (Fig. 1).

Identification of PSD31 as an adenine nucleotide translocator: The present elution protocol was applied for sequencing a 31 kDa protein in the PSD fraction. The 31 kDa protein (termed PSD31), which was mostly insoluble in 1% OG (Fig. 2, asterisk), was electroeluted from 10% preparative gels, digested by trypsin directly on gel, and tryptic peptides were purified by HPLC. Both of the two peptides submitted for sequencing were successfully determined for amino acid sequences. The amino acid sequences were highly homologous with the rat adenine nucleotide translocator 1 (ANT1; GenBank accession number Q05962) [18] (Table 1). The authenticity of the peptides was verified by the presence of methiones (M) at the -1 positions of the CNBr-cleaved peptides and
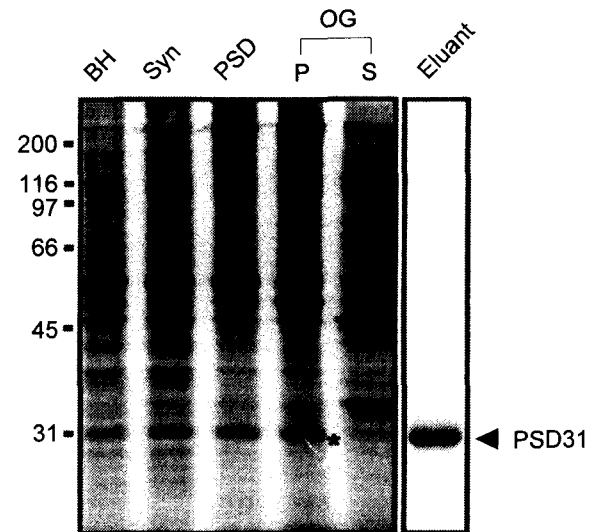

Fig. 2. Fractionation of the PSD proteins and the target protein PSD31.

Forty micrograms of each One-Triton, OG-S and OG-P fractions were separated in a 10% SDS-gel, stained with Coomassie dye and destained as usual. The PSD31, the target protein sequenced in this work, was marked as an asterisk in the OG-P lane. Electroeluted PSD31 was marked. Molecular sizes are indicated at left in kilodaltons (kDa).

arginines (R) or lysine (K) bracketing the tryptic peptides [14] (Table 1).

Discussion

In this study, we described an efficient and reliable electroelution protocol, showed that it could be applicable for extracting proteins with diverse molecular sizes from SDS-gels, and succeeded in obtaining internal amino acid sequences of a 31 kDa proteins in the OG-insoluble PSD fraction identifying it as ANT1, or its homologue.

The electrophoretic profile of PSD is very complex. If proteins are fixed in the gel during staining with Coomassie dye, elution efficiency is usually very low or it takes a long time (> 8h) to get an satisfactory efficiency [7]. Other possible options are to negatively stain the proteins with sodium acetate [6] or cupper

Table 1. Amino Acid Sequences.

Peptides	Amino acid Sequences
peptide 1	GFLANVIR
	* * * * * * *
ANT1 (73-80)	GNLANVIR
peptide 2	AAYFGVYDTAK
	* * * * * * * * * * *
ANT1 (189-199)	AAYFGVYDTAK
peptide 3	AFFK
	* * * *
ANT 1 (268-271)	AFFK

chloride [12]. Unfortunately, these alternative methods result in a poor resolution and difficult to apply for a complex electrophoretic profile such as PSD. The protocol employed in this study is based on the assumption that proteins stained with Coomassie dye in a buffer that lacks fixatives could be visualized unambiguously and also the stained proteins could be efficiently eluted since they are not fixed to the gel matrix. As expected, the present protocol resulted in a good resolution that allowed unambiguous identification of major protein bands (approximately over 1% of the total fraction), and elution efficiencies were reasonably high (60-80% for most of the PSD proteins), irrespective of their molecular sizes. It should be also noted that the calculated elution efficiency must be underevaluated, since the background smear in the PSD lane was much higher than in sample lanes.

The present elution method was successfully applied for sequencing a 31 kDa protein in the OG-insoluble fraction of the PSD. The amino acid sequence information and similarity in molecular sizes indicate that PSD31 is a homolog of rat adenine nucleotide translocator 1 (ANT1 or ATP/ADP translocase 1) [18,20]. The ANT1 has been known to catalyze the exchange of ADP and ATP across the mitochondrial internal membrane [2,10], and, so far, no extramitochondrial ANT1 have been reported except in higher-plant plastids [21,22]. Although a possibility for mitochondrial contribution of ANT1 to the PSD fraction can not be ruled out, it is unlikely by the following reasons. First, the ANT1 is an abundant protein representing ~3% of the total NOG-P proteins. Second, the ANT1 is resistant to extraction by Triton X-100 during One-Triton PSD preparation and by NOG, indicating strong association of the ANT1 with PSD. The function of the ANT1 in PSD is highly speculative at present. Extracellular ATP has been well characterized as a signaling molecule in diverse cell types including neurons [4,5,24]. However,

how ATP is released to extracellular space is elusive. The PSD-associated ANT1 may play a role in releasing ATP into the synaptic cleft. Further studies employing confocal and immunoelectron microscopes are necessary for the precise localization of ANT1 in the synapse.

Acknowledgment

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Jae-Seob Jung1, Sun-Jung Cho, Seung Chul Shin, Ingnyol Jin, Yong Wook Jung, Bok Hyun Ko, Il Soo Moon

초록 : SDS-겔로부터 효율적인 단백질 분리 방법과 31 kDa 연접후치밀질 단백질의 동정

정재섭[1] · 조선정[1] · 신승철[2] · 진익렬[1] · 정용욱[2] · 고복현[2] · 문일수[2*]

([1]경북대학교 자연과학대학 미생물학과, [2*]동국대학교 의과대학 해부학교실)

연접후치밀질(PSD)의 분자조성은 아직 대체로 알려져 있지 않다. 본 연구에서는 다양한 크기의 연접후치밀질 단백질들을 효율적으로 SDS-겔에서부터 추출하는 방법을 제시하고, 이를 이용하여 연접후치밀질의 1% n-octyl glucoside에 용해되지 않는 분핵에 존재하는 31 kDa 크기의 단백질(PSD31로 명명)을 SDS-겔로부터 분리하고, trypsin 처리하여 생성된 펩티드의 아미노산 서열을 결정하였다. PSD31의 아미노산 서열은 adenine nucleotide translocator 1 (ANT1)과 매우 상동성이 높았다. 연접후치밀질에 ANT1이 존재함은 신경조직의 세포외 공간에 adenosine nucleotide를 유리할 수 있는 기구가 연접에 존재함을 시사한다.