Poly(ADP-ribose) Preparation Using Anion-Exchange Column Chromatography

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Abstract: Poly(ADP-ribose) polymerase (PARP) polyADP-ribosylates proteins involved in various physiological processes. Accumulated evidence suggests not only protein-conjugated poly(ADP-ribose) but also protein-free poly(ADP-ribose) function in various physiological processes. There are increasing occasions that require protein-free poly(ADP-ribose) to study the function and dynamics of poly(ADP-ribose) in cells. However, the availability of poly(ADP-ribose) is still limited because a chemical synthesis method has not been established. Here, we describe an improved method for the preparation of protein-free poly(ADP-ribose), synthesized enzymatically by using a recombinant PARP-1 expression system and purified with an anion-exchange column chromatography. This method will be useful for biochemical and biological investigation of poly(ADP-ribose) functions and dynamics.

Keywords: poly(ADP-ribose) polymerase, poly(ADP-ribose), anion-exchange column, preparation

Introduction

Poly(ADP-ribose) is a biomacromolecule produced in a polyADP-ribosylation reaction, one of the post-translational modifications of proteins.[1] The involvement of PARP-1 in development of insulin-dependent diabetes mellitus[2–4] cerebral ischemia[5] and in maintenance of genomic stability[6,7] has been reported. The presence of poly(ADP-ribose) polymerase family proteins[8–10] has been reported and proteins which bind poly(ADP-ribose) have been described.[11–14] Understanding the role of protein-conjugated and released poly(ADP-ribose) molecules has become a more important issue than ever.

Preparation of poly(ADP-ribose) polymer is necessary for studying polyADP-ribosylation and poly(ADP-ribose) metabolism. Unlike DNA or RNA, chemical synthesis of poly(ADP-ribose) is still not practical and enzymatic synthesis of poly(ADP-ribose) is being carried out. Small scale preparation of radioisotopically-labelled or unlabelled poly(ADP-ribose) has been carried out. Using extracts of cultured cells or tissues such as calf thymus, poly(ADP-ribose) synthesis reaction was carried out and after detachement of protein and removal of protein, DNA and RNA, poly(ADP-ribose) was purified with a molecular sieve column, hydroxylapatite column, ion-exchange column or an affinity-column chromatography with boronate resin. Molecular sieve columns such as Zorvax G450 (Dupont) and HPLC column TSK-125 (Bio-Rad) have been used,[15] Boronate resins, such as dihydroxyboryl Bio-Rex 70 (Bio-Rad) have an affinity with cis-diol groups and have been also used for preparation of poly(ADP-ribose). Elution of poly(ADP-ribose) is carried out with diluted HCl.[16] The size fractionation of poly(ADP-ribose) is not possible with boronate resin. Ion-exchange column for HPLC has been applied for purification of oligo- and poly(ADP-ribose).[17] Another frequently used procedure for the purification of poly(ADP-ribose) involves hydroxylapatite column chromatography separation.[18] This step can remove short DNA or RNA fragments. It also allows rough size-dependent elution of poly(ADP-ribose). However, size fractionation of poly(ADP-ribose) cannot be achieved by this procedure. In addition, elution with high concentrations of phosphate buffer requires further exclusive dialysis to remove phosphates.

To improve the purification method of poly(ADP-ribose), we utilized QIAGEN-tip (Qiagen, Hilden, Germany), which is commercially available for plasmid DNA purification.[19] This column contains pre-packed QIAGEN resin, which has a hydrophilic surface coated with diethylaminoethyl groups.

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Poly(ADP-ribose) can be eluted with a buffer containing NaCl, which is easily removed by dialysis. Furthermore, this column allows better size separation of the polymer. This method was only briefly mentioned in our previous reports[20,21] and here we describe the detailed procedure and the characterization of the prepared poly(ADP-ribose). The procedure described here will be useful for researchers who study poly(ADP-ribose) function.

Materials and Methods

Poly(ADP-ribose) was prepared using an *E. coli* crude extract that overexpressed human PARP-1, as described.[22] The *E. coli* crude extract from one liter contained approximately 72 units of PARP activity (one unit of PARP activity was defined as 1 nmol ADP-ribose incorporation per minute at 25 °C). Briefly, pellets of *E. coli* expressing PARP-1 from a 4-liter culture (approximately 10 ml) were suspended in 20 ml of lysis buffer-200 μg/ml lysozyme and incubated at 0 °C for 15 min, then NaCl and NP-40 were added to 0.6 M and 1.0%, respectively. A supernatant was obtained by ultracentrifuge at 39,800 *g* for 60 min at 4 °C, desalted with Centriprep (Millipore, Billerica, Massachusetts, U.S.A.) and was subjected to poly(ADP-ribose) synthesis reaction. A reaction mixture of 50 ml containing the above lysate, 30 mM $MgCl_2$, 20 μg/ml histone (Sigma-Aldrich Corp.), 10 μg/ml activated calf thymus DNA (Sigma-Aldrich Corp.), 1 mM NAD, and proteinase inhibitor cocktail (Complete, Roche Applied Science, Mannheim, Germany) was prepared and incubation was carried out for 60 min at 25 °C. The reaction was stopped by adding 1/50 volume of 5 N NaOH, and further incubated for 60 min at 37 °C to release poly(ADP-ribose) from acceptor proteins.

After addition of Tris-HCl (pH 7.5) to 50 mM, the pH was adjusted to 7.5 with HCl, and incubated overnight after addition of SDS to 0.1% and proteinase K (Merck and Co., Inc., Readington Township, New Jersey, U.S.A.) to 50 μg/ml at 37 °C in a volume of approximately 50 ml. Protein was removed by extracting with equal volumes (50 ml) of water-saturated phenol/chloroform (1:1 (v/v)), mixing vigorously for 10 min, centrifuging at 1,500 *g* at room temperature and taking the supernatant. The sample was then extracted with equal volumes of chloroform two times, and the aqueous layer obtained after centrifugation was processed to ethanol precipitation. To the aqueous layer, 1/10 volume of 3 M sodium acetate (pH 5.0) and 2 volumes of ethanol were added. The sample was kept at −80 °C for 15 min and centrifuged at 10,000 *g* for 15–20 min at 4 °C and the pellet was taken. After washing the pellet with 30 ml of 70% ethanol and drying, the pellet was resuspended in 20 ml of a buffer consisting of 50 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, 5 mM $CaCl_2$, 4 μg/ml DNase I (Takara Bio Inc., Otsu, Shiga, Japan), 4 μg/ml RNase A (Sigma-Aldrich Corp.), 10 μg/ml nuclease P1 (Yamasa Corp., Choshi, Chiba, Japan), and 1% toluene and incubated overnight at 37 °C. After addition of pronase E (Sigma-Aldrich Corp.) to 0.1 mg/ml, incubation was continued at 37 °C for 2 hr. Following extraction with equal volumes of water-saturated phenol/chloroform 1:1(v/v) for 10 min by mixing vigorously, as described above, the sample was centrifuged at 1,500 g at room temperature and the supernatant taken. The sample was then extracted with equal volumes of chloroform two times, and the aqueous layer obtained after centrifugation was processed to ethanol precipitation. Poly(ADP-ribose) was recovered from the aqueous layer by ethanol precipitation by adding ammonium acetate (pH 7.0) to 2 M and adding 2.5 volumes of ethanol at room temperature. After centrifugation at 3,000 *g* for 15 min at 4 °C, washing the pellet with 70% ethanol and drying, the pellet was resuspended into 10 ml of a solution consisting 50 mM Tris-HCl (pH 7.0) and 10 mM EDTA.

A column of QIAGEN-tip 500 was equilibrated with 10 ml of buffer QBT [750 mM NaCl, 50 mM MOPS (pH 7.0), 15% ethanol, 0.15% Triton X-100], and allowed to empty by gravity flow.[19] Ten ml of poly(ADP-ribose) solution was applied to the QIAGEN-tip with gravity flow. After washing with 2 × 10 ml wash buffer [50 mM MOPS (pH 7.0), 0.4 M NaCl, 15% ethanol], stepwise elution of poly(ADP-ribose) was conducted with 10 ml of elution buffer-1 [50 mM MOPS (pH 7.0), 0.6 M NaCl, 15% ethanol], 10 ml of elution buffer-2 [50 mM MOPS (pH 7.0), 0.8 M NaCl, 15% ethanol], 10 ml of buffer QC [50 mM MOPS (pH 7.0), 1.0 M NaCl, 15% ethanol] and 10 ml of buffer QF [50 mM MOPS (pH 7.0), 1.2 M NaCl, 15% ethanol]. Ethanol in each buffer can be substituted with propanol. Washing and elution buffers were degassed or autoclaved to shorten the chromatographic steps. Each fraction was analyzed for poly(ADP-ribose) chain length distribution by 20% polyacrylamide gel electrophoresis as described,[23]

and dialyzed against 3 liters of water at room temperature overnight. The desalting step could be replaced by ethanol or isopropanol precipitation. Capillary electrophoresis was performed using ssDNA Gel Linear Polyacrylamide with Tris-borate-7 M urea (P/ACE, Beckman Coulter Inc., Fullerton, California, U.S.A).

For preparation of ^{32}P-labelled-poly(ADP-ribose), an *E. coli* pellet from a 500 ml culture was prepared and 1–3 volumes of lysis buffer [50 mM Tris-HCl (pH 8.0), 1 mM dithiothreitol, 1 mM phenylmethanesulfonyl fluoride, 10 μg/ml leupeptin] were added. Egg white lysozyme was added to 200 μg/ml and incubated at 0 °C for 15 min. After adding NaCl to 0.6 M and NP-40 to 1.0%, centrifuge was carried out for 45 min at 25,000 *g* at 4 °C. The supernatant, approximately 5 ml, was added to a 50 ml reaction mixture containing 50 mM Tris-Cl (pH 7.5), 1 mM dithiothreitol, 30 mM $MgCl_2$, 20 μg/ml histone (Sigma-Aldrich Corp.), 10 μg/ml activated calf thymus DNA (Sigma-Aldrich Corp.), 10–20 μM [adenylate-^{32}P] NAD (0.46 MBq/nmol, NEN, PerkinElmer, Inc., Waltham, Massachusetts, U.S.A.), proteinase inhibitor cocktail (Complete) and incubation was carried out for 60 min at 25 °C. This reaction was stopped with 1/50 volume of 5 N NaOH, and further incubated for 60 min at 37 °C to release poly(ADP-ribose) from acceptor proteins and processed as described above for preparation of cold poly(ADP-ribose). Either QIAGEN-tip 500 or -tip 100 was used for preparation of ^{32}P-poly(ADP-ribose) as described above.

For determination of the size of ^{32}P-poly(ADP-ribose), hydrolysis by purified venom phosphodiesterase was carried out. Phosphodiesterase from *Crotalus adamanteus* (Worthington Biochemical Corp., Lakewood, New Jersey, U.S.A.) was purified by a Blue-Sepharose column to remove any traces of phosphomonoesterase.[24,25] The sample was digested in a reaction mixture containing 10 mM sodium phosphate buffer (pH 7.0), 10 mM $MgCl_2$, and 0.004 unit of the purified phosphodiesterase[24] and incubated for 3 hr or overnight. The samples were spotted on F1440 (Scheleicher and Schuell, Dassel, Germany) TLC plate and developed with solvent A: isobutyric acid/25% NH_4OH/H_2O (50/1.1/28.9 by volume), and B: 0.1 M sodium phosphate (pH 6.8)/ammonium sulfate/n-propanol (100/60/2, v/w/v). The radioactivity of each spot was analyzed by BAS2000 (Fujifilm, Minato-ku, Tokyo, Japan). Each spot was characterized as AMP, phosphoribosyl-AMP (PR-AMP), diphosphoribosyl-AMP ($(PR)_2AMP$). The average polymer size was calculated as [AMP + PR-AMP + $(PR)_2AMP$]/[AMP – $(PR)_2AMP$] as described.[26]

Results and Discussion

As shown in Figure. 1A, a rapid and size separation of ^{32}P-poly(ADP-ribose) was achieved with the QIAGEN-tip column by changing the pH and salt concentration of the elution buffer. The average polymer size and the average number of branching points were determined by two-dimensional

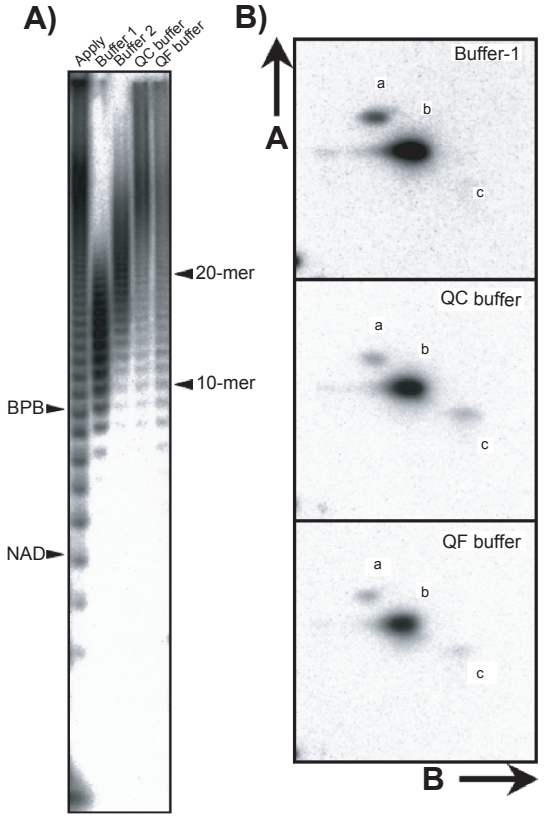

Figure. 1. A) ^{32}P-Poly(ADP-ribose) fraction on QIAGEN-tip. 20% polyacrylamide gel electrophoresis analysis. One μl of samples of each fraction was mixed with 20 μl of sample buffer [50% urea, 25 mM NaCl, 4 mM EDTA, 0.02% xylene cyanol, 0.02% bromophenol blue], and applied to 20% polyacrylamide gel (acrylamide/bisacrylamide, 19.85/0.25 by weight).[23] Applied fraction (apply), eluted fractions with buffer 1, buffer 2, QC buffer and QF buffer were subjected to analysis. The radioactivity of the gel was analyzed by BAS2000. **B**) Two-dimensional TLC for characterization of ^{32}P-poly(ADP-ribose). After hydrolysis by purified venom phosphodiesterase, the samples (eluent with buffer-1, QC buffer and QF buffer) were spotted on a TLC plate and developed with solvent A and B as described in Materials and Methods. The radioactivity of each spot was analyzed by BAS2000. Each spot was characterized as AMP ((a), phosphoribosyl-AMP (PR-AMP) (b), and diphosphoribosyl-AMP ($(PR)_2AMP$) (c).

thin-layer chromatography after phosphodiesterase treatment[26] as shown in Figure. 1B. The typical yield of cold poly(ADP-ribose) prepared from 4 liters of lysate of the *E. coli* was 0.393 μmol (ADP-ribose residues, 216 μg), 1.419 μmol (780 μg), 0.156 μmol (86 μg) for buffer-1, QC and GF, respectively. Mean chain-length in each fraction was 17-mer, 20-mer, 26-mer, for buffer-1, QC buffer and QF buffer, respectively. Branched poly(ADP-ribose) could be observed in the fractions eluted with QC and QF. It should be noted that doublet bands were observed as reported by Panzeter et al. due to two different hydrolysis products at the reducing termini.[23] The maximum capacity of QIAGEN-tip 100 and -tip 500 for DNA is around 100 μg and 500 μg, respectively, according to the manufacturer's recommendation. The above result suggests maximum capacity of QIAGEN-tip 500 for poly(ADP-ribose) is approximately 1,000 μg or higher. Figure. 2A shows non-radioisotopic poly(ADP-ribose) prepared using the same method. Figure. 2B shows the capillary electrophoresis pattern of the poly(ADP-ribose). This method should be applicable for rapid and larger scale preparation of poly(ADP-ribose).

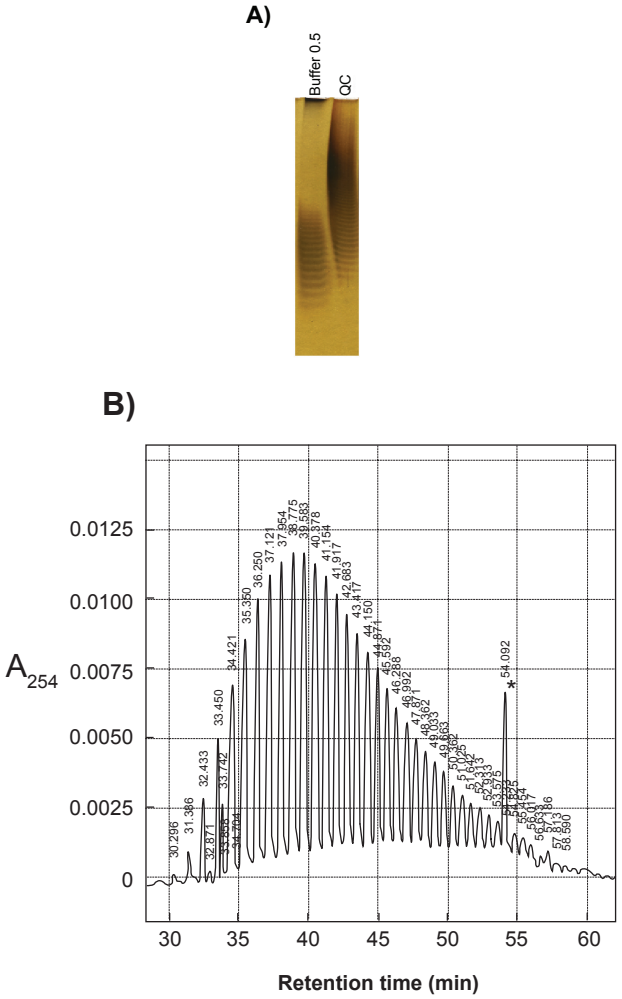

Figure. 2. A) Analysis of poly(ADP-ribose) by 20% polyacrylamide gel electrophoresis. Twenty μg of buffer 0.5 [50 mM MOPS (pH 7.0), 0.5 M NaCl, 15% propanol] eluted fraction and QC buffer [50 mM MOPS (pH 7.0), 1.0 M NaCl, 15% propanol] eluted fraction were applied. Note that ethanol in the buffers was substituted with propanol. After electrophoresis, the gel was subjected to silver-staining (Daiichi Pure Chemicals Co. Ltd., Chuo-ku, Tokyo, Japan). **B**) Capillary electrophoresis profile of poly(ADP-ribose). The fraction eluted with QC buffer was subjected to capillary electrophoresis. Detection was carried out with measurement of UV absorbance at 254 nm. The retention time of each peak is shown. A peak marked with an asterisk is derived from an unknown UV absorbing substance in the preparation.

Abbreviations

PARP, poly(ADP-ribose) polymerase; NAD, nicotinamide adenine dinucleotide.

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Disclosure

The authors report no conflicts of interest.

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