ELSEVIER

Journal of Chromatography A, 678 (1994) 35–40

JOURNAL OF CHROMATOGRAPHY A

Fast protein liquid chromatographic purification of poly(ADP–ribose) polymerase and separation of ADP–ribose polymers

Phyllis L. Panzeter, Barbara Zweifel, Felix R. Althaus*

Institute of Pharmacology and Toxicology, University of Zürich–Tierspital, Winterthurerstrasse 260, CH-8057 Zurich, Switzerland

(First received March 14th, 1994; revised manuscript received May 26th, 1994)

Abstract

Poly(ADP–ribose) polymerase responds to DNA strand breaks in nuclei by producing ADP–ribose polymers covalently attached to proteins. Here we report two fast protein liquid chromatographic applications to aid investigations on poly(ADP–ribosyl)ation. The first rapidly purifies poly(ADP–ribose) polymerase from crude calf thymus extract. The purification protocol, involving successive fractionations over four columns, reduces the time for polymerase purification from four days to 14 h resulting in a >50% increase in enzyme-specific activity. The second application employs a complex salt gradient to reproducibly separate ADP–ribose polymers into individual size classes.

1. Introduction

Poly(ADP–ribosyl)ation is required for repair of DNA breaks in higher eukaryotes [1–3]. Poly(ADP–ribose) polymerase (EC 2.4.2.30) is dependent on DNA strand breaks for activation [4], and activation in turn leads to modification of polymerase molecules with long polymers of ADP–ribose (automodification). Through non-covalent interactions with histones [5–7], these polymers disrupt DNA–histone complexes making the DNA accessible to DNA processing enzymes [8,9]. Degradation of ADP–ribose polymers by poly(ADP–ribose) glycohydrolase restores the integrity of the DNA–histone complex. Polymer size and structure play an important role in this histone shuttle mechanism, influencing both polymer affinity for chromatinic proteins [6] and polymer degradation kinetics [10,11]. We therefore found it necessary to prepare ADP–ribose polymers of distinct sizes to further elucidate the histone shuttle of chromatin.

We have established two fast protein liquid chromatographic (FPLC; Pharmacia) applications to aid our investigations. (1) Because purification of poly(ADP–ribose) polymerase requires successive fractionation of a calf thymus crude extract over four different chromatography resins [12–14], conventional chromatography techniques take at least four days before the pure enzyme is obtained. Strategic programming and continuous flow from column to column using FPLC reduced purification time to 14 h thereby yielding more active enzyme faster. (2) Separation of ADP–ribose polymers into indi-

* Corresponding author.

0021-9673/94/$07.00
SSDI 0021-9673(94)00487-T

vidual size classes has recently been achieved using HPLC [15]. We have found that high-resolution separation of polymers by FPLC using a MonoQ column is highly reproducible and can process large quantities of polymers with recoveries of 98 ± 7%.

2. Materials and methods

2.1. Poly(ADP–ribose) polymerase purification

Preparation of calf thymus crude extract

Frozen calf thymus (50 g) was homogenized in 250 ml of 50 m*M* Tris, 0.3 *M* NaCl, 10% glycerol, 10 m*M* β-mercaptoethanol (β-ME), 50 m*M* $Na_2S_2O_5$, pH 8.0. After centrifugation at 12 000 *g* for 15 min at 4°C, the supernatant was precipitated with 30% $(NH_4)_2SO_4$, centrifuged, and reprecipitated with 70% $(NH_4)_2SO_4$. The resulting pellet was resuspended in 10 ml basis buffer (100 m*M* Tris–HCl, 17% glycerol, 25 m*M* $K_2S_2O_5$, 12 m*M* β-ME, 0.5 m*M* EDTA) and loaded into a 50-ml Superloop (Pharmacia).

Columns and FPLC configuration

DNA-cellulose (Pharmacia) was nicked [16] and packed in an HR 10/30 column (Pharmacia). 3-Aminobenzamide was cross-linked to AffiGel 10 (Bio-Rad) and packed in an HR 10/30 column. A 0.2-ml volume of hydroxyapatite (HTP Bio-Gel; Bio-Rad) was prepared fresh for each purification and packed in a 10-ml EconoColumn (Bio-Rad). FPLC (with LCC-500 Plus controller; Pharmacia) connections to columns, valves and buffers are schematically shown in Fig. 1. The system was run at 4°C; the flow-rate was maintained at 0.4 ml/min. For

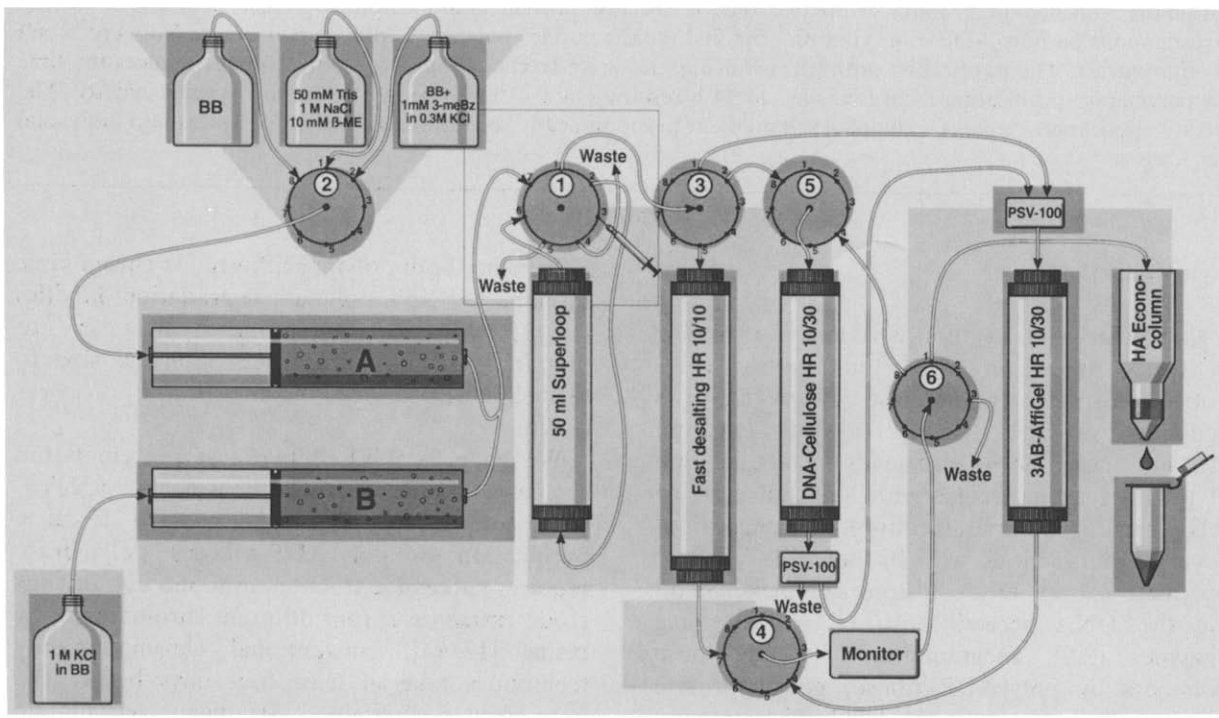

Fig. 1. Schematic diagram of FPLC system for purification of poly(ADP–ribose) polymerase. Purification of poly(ADP–ribose) polymerase from calf thymus crude extract requires successive fractionation over Sephadex G-25, DNA–cellulose, 3-aminobenzamide–AffiGel and hydroxyapatite columns. Using the basic FPLC system with strategic programming of the valves (1–6), the time needed for polymerase purification was reduced from four days to 14 h. 3-meBz = 3-Methoxybenzamide.

further details, a copy of the run program is available on request.

Enzyme activity assay

The specific activity of poly(ADP–ribose) polymerase was calculated from the amount of NAD^+-derived ADP–ribose incorporated into acid-precipitable material. A 10-μl volume of the solution to be assayed was added to 50 m*M* Tris (pH 8.0), 1 m*M* dithiothreitol, 10 m*M* $MgCl_2$, 2 μg nicked calf thymus DNA [16], 2 μg H1 (Boehringer Mannheim) and 10 μM [^{3}H]NAD^+ (45 Ci/mol; New England Nuclear) in a final volume of 100 μl. After incubation at 25°C for 10 min, samples were precipitated with 20% trichloroacetic acid, applied to glass fiber filters, washed with 5% trichloroacetic acid, and counted for radioactivity.

2.2. Separation of ADP–ribose polymers

Polymer synthesis and purification

Protein-attached polymers of [^{32}P]ADP–ribose were synthesized in a 9-ml reaction mix containing 3 mg of crude poly(ADP–ribose) polymerase (430 pmol/min mg), 50 m*M* Tris (pH 8.0), 1 m*M* dithiothreitol, 10 m*M* $MgCl_2$, 100 μg nicked calf thymus DNA [16], 225 μg H1, 9.6% ethanol and 1 m*M* [^{32}P]NAD^+ (10 Ci/mol; New England Nuclear). After 30 min at 25°C, protein-attached polymers were precipitated with 20% trichloroacetic acid, dissolved in 98% formic acid, and reprecipitated with trichloroacetic acid.

Each pellet was resuspended in 1 ml of 1 *M* KOH/50 m*M* EDTA and incubated at 37°C for 2 h to detach [^{32}P]poly(ADP–ribose) from protein. Conditions were adjusted to pH 8 and 50 m*M* $MgCl_2$ whereupon DNA was digested with 1000 U of DNase I (Sigma) for 2 h at 37°C. Proteins were subsequently digested with 200 U of proteinase K (Boehringer Mannheim) at 37°C overnight. After extraction with an equal volume of phenol–$CHCl_3$–isoamyl alcohol (49:49:2), [^{32}P]poly(ADP–ribose) was precipitated with ethanol and dried in a Speed-Vac concentrator. The polymers of [^{32}P]ADP–ribose were dissolved in water and stored at −20°C.

Columns and FPLC configuration

A 1-ml MonoQ column (Pharmacia) was used at a flow-rate of 0.4 ml/min; 0.4-ml fractions were collected. Gradient buffer A contained 20 m*M* Tris, pH 8.3, and buffer B consisted of 1 *M* KCl in buffer A. The system was run at 4°C.

High-resolution size analysis of polymers

Fractions from FPLC separation were counted for ^{32}P content. Aliquots containing 100 dpm from each peak fraction were dried, dissolved in 10 μl of loading buffer and separated on a polyacrylamide gel as previously described [17].

3. Results and discussion

Strategic design and programming of FPLC and reproducible high resolution from FPLC columns have allowed us to not only rapidly purify the nuclear enzyme poly(ADP–ribose) polymerase but also to resolve its polymeric ADP–ribose products. The significant attributes of each application are discussed below.

3.1. Poly(ADP–ribose) polymerase purification

Purification of poly(ADP–ribose) polymerase requires successive fractionation of a crude extract over four chromatography resins, three of which are affinity resins. Previous purification protocols required many technical manipulations in a cold room over a period of four days. Using FPLC, we have completely automated the column chromatography fractionations such that the technician need only perform a preparative ammonium sulfate precipitation and load the crude extract onto the FPLC. The new procedure yields pure poly(ADP–ribose) polymerase in only 14 h.

The FPLC setup is schematically shown in Fig. 1. Notable variations from usual setups include the use of a valve (valve 2) for selection of elution buffers, a valve before the UV monitor (valve 4) to select which eluent to monitor, and the use of PSV-100 valves to direct flow to/from columns. The FPLC system itself resides in a

cold room and is connected serially to the computer driver in a nearby office. Once the crude extract is loaded onto the FPLC system, all FPLC manipulations and monitoring of results are done at the computer station.

After preparation of the crude extract (see Materials and methods), the sample containing 200–300 mg of protein is loaded into the Superloop and the FPLC program initiated. Protein is desalted and automatically loaded onto nicked DNA–cellulose. Poly(ADP–ribose) polymerase binds with high affinity to DNA nicks [18] and is subsequently eluted with a 0.2–1 *M* KCl linear gradient in basis buffer. The polymerase elutes in a sharp peak at about 0.8 *M* KCl and is shunted directly to a 3-aminobenzamide AffiGel column. After four column washes, the enzyme is competitively eluted from the AffiGel resin with basis buffer containing 0.3 *M* KCl/1 m*M* 3-methoxybenzamide and is concentrated and washed on a 0.2-ml pad of hydroxyapatite. Elution of poly(ADP–ribose) polymerase from hydroxyapatite is performed manually with two 250-μl aliquots of basis buffer containing 0.5 *M* potassium phosphate, pH 7.2.

A comparison of purification parameters from the conventional procedure versus FPLC is given in Table 1. While the overall yields are comparable, a 53% increase in enzyme-specific activity was obtained using FPLC mainly due to the decreased processing time. Like the conventional preparation, FPLC-purified poly(ADP–ribose) polymerase contains no detectable DNA topoisomerase activity and is electrophoretically pure (Fig. 2).

Table 1
Purification of poly(ADP-ribose) polymerase from calf thymus using conventional chromatography versus FPLC

	Conventional chromatography	FPLC
Protein recovery (%)	5.2	5.8
Specific activity (nmol/min mg)	378	578
Purification (x-fold)	1026	1399

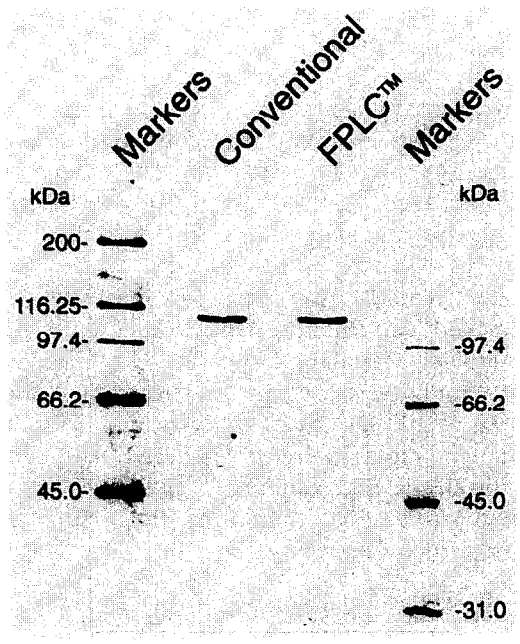

Fig. 2. Gel analysis of purified poly(ADP–ribose) polymerase. A 1-μg amount of protein prepared by conventional chromatography techniques or FPLC was separated on a 10% sodium dodecyl sulphate–polyacrylamide gel [20] and stained with Coommassie Blue. Markers with molecular masses (kDa = kilodalton) as indicated were loaded in the first and last lanes.

3.2. Separation of ADP–ribose polymers

Poly(ADP–ribose) polymerase synthesizes polymers of NAD^+-derived ADP–ribose, the sizes of which respond to the protein environment at the time of synthesis [19]. It has been difficult to further analyze the specific role of discrete polymer sizes since homogeneous polymer size classes could not be isolated. We have overcome this obstacle using FPLC.

After synthesis and detachment from protein (see Materials and methods), 50–100 nmol of [^{32}P]ADP–ribose in the form of polymers were injected in 0.5 ml of buffer A onto a 1-ml MonoQ column. The polymers were eluted with a KCl gradient (caption to Fig. 3) and fractions collected. A typical elution profile is shown in Fig. 3. As polymer size increases, the elution

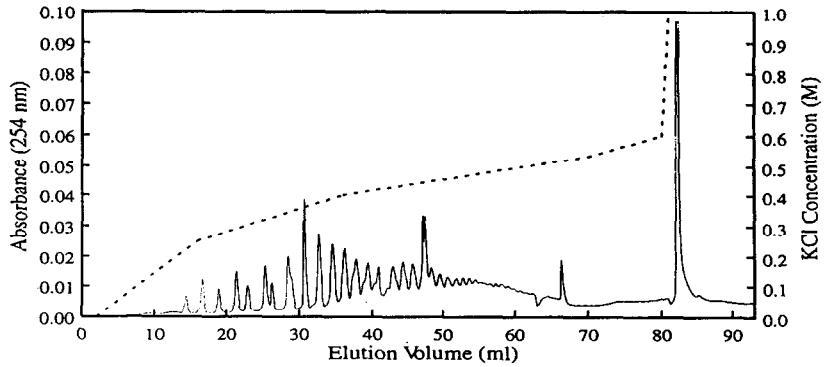

Fig. 3. Separation of ADP–ribose polymers by FPLC. Polymers of ADP–ribose in a total volume of 0.5 ml were injected onto a 1-ml MonoQ column. Absorbance (solid line) was monitored continuously during elution with a KCl gradient (broken line). The gradient program used was: 0% buffer B at 0 ml, 0% B at 2 ml, 25% B at 16 ml, 40% B at 36 ml, 53% B at 70 ml, 60% B at 80 ml, and 100% B at 81 ml (see also Materials and methods).

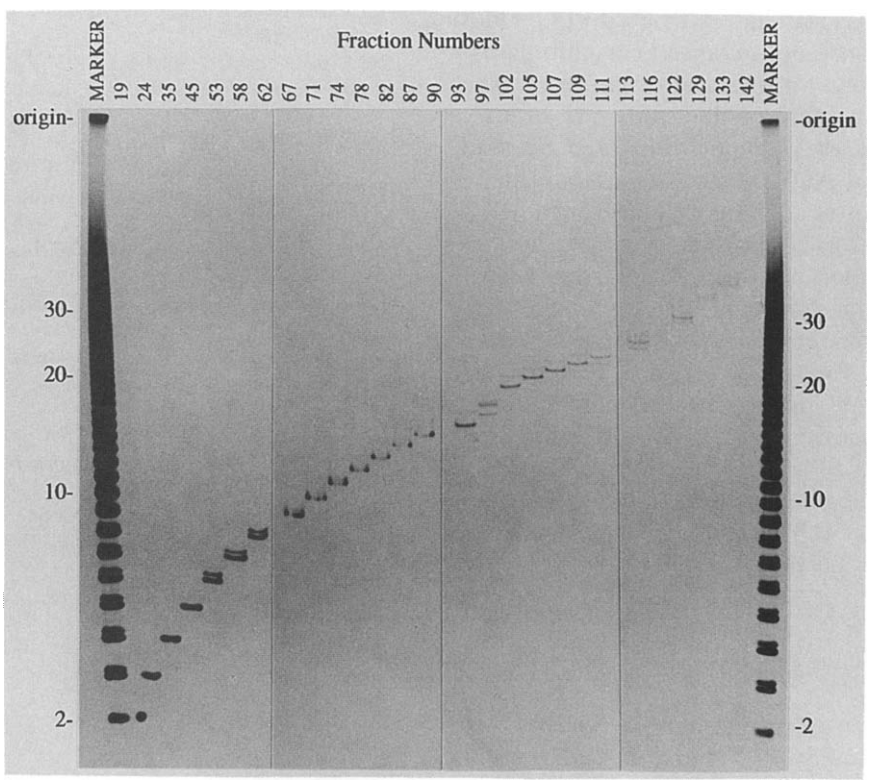

Fig. 4. Poly(ADP–ribose) size analysis following FPLC separation. A heterogeneous population of [^{32}P]ADP–ribose polymers (MARKER) was separated on a 1-ml MonoQ column and fractions collected (see Materials and methods). Aliquots containing 100 dpm of [^{32}P]poly(ADP–ribose) were analyzed on high-resolution polyacrylamide gels and subjected to autoradiography [17]. The lengths of the polymers in terms of ADP–ribose residues are indicated to the left and right of the autoradiograph.

gradient becomes more shallow to maximize resolution. Aliquots from peak fractions were analyzed by autoradiography of high-resolution polymer gels [17]. Fig. 4 shows that the MonoQ resin easily resolved polymers ranging from 2–24 ADP–ribose units and resolved polymers ranging from 26–50 ADP–ribose residues by units of 2–4. In addition, polymers eluting in 1 *M* KCl did not migrate upon electrophoretic analysis (not shown) and therefore may represent branched polymers [6,15]. Overall recovery of radioactivity from the MonoQ was always $>90\%$.

Recently, Kiehlbauch et al. [15] have reported the separation of polymers on a Progel-TSK DEAE NPR HPLC column. While separation of polymers using HPLC was comparable to FPLC, they cited potential problems with reproducibility dependent on the HPLC system used. Supplied as a standard system, FPLC eliminates such variabilities. Also unlike HPLC, FPLC lends itself to the scale-up of analytical chromatography separations for preparative purposes. The MonoQ column used for this study has an ionic capacity of 0.27–0.37 mmol which is equivalent to 75–100 mg of ADP–ribose. Separation can be easily scaled up to a 20-ml column which could separate up to 2 g of ADP–ribose polymers. This becomes an important aspect for polymer preparation when one considers that, in a heterogeneous polymer population, a single size class of polymers represents only one of at least 50 size classes, and the longer the polymer, the less frequent its occurrence [17]. For these reasons, we recommend FPLC for the large-scale preparation of homogeneous ADP–ribose polymer populations as well as for poly(ADP–ribose) polymerase purification.

Acknowledgements

This work was supported by grants (to F.R.A.) from the Swiss National Foundation for Scientific Research (31.31203.91), the Krebsliga des Kantons Zürich, and the Jubiläumsstiftung der Universität Zürich.

References

[1] F.R. Althaus and C. Richter, *Mol. Biol. Biochem. Biophys.*, 37 (1987) 1.
[2] J.E. Cleaver and W.F. Morgan, *Mutation Res.*, 257 (1991) 1.
[3] M.S. Satoh and T. Lindahl, *Nature*, 356 (1992) 356.
[4] R.C. Benjamin and D.M. Gill, *J. Biol. Chem.*, 255 (1980) 10502.
[5] J. Wesierska-Gadek and G. Sauermann, *Eur. J. Biochem.*, 173 (1988) 675.
[6] P.L. Panzeter, C.A. Realini and F.R. Althaus, *Biochemistry*, 31 (1992) 1379.
[7] P.L. Panzeter, B. Zweifel, M. Malanga, S.H. Waser, M.C. Richard and F.R. Althaus, *J. Biol. Chem.*, 268 (1993) 17662.
[8] C.A. Realini and F.R. Althaus, *J. Biol. Chem.*, 267 (1992) 18858.
[9] F.R. Althaus, *J. Cell Sci.*, 102 (1992) 663.
[10] K. Hatakeyama, Y. Nemoto, K. Ueda and O. Hayaishi, *J. Biol. Chem.*, 261 (1986) 14902.
[11] S.A. Braun, P.L. Panzeter, M.A. Collinge and F.R. Althaus, *Eur. J. Biochem.*, 220 (1994) 369.
[12] S. Ito, Y. Shizuta and O. Hayaishi, *J. Biol. Chem.*, 254 (1979) 3647.
[13] S.G. Carter and N.A. Berger, *Biochemistry*, 21 (1982) 5475.
[14] B. Kofler, E. Wallraff, H. Herzog, R. Schneider, B. Auer and M. Schweiger, *Biochem. J.*, 293 (1993) 275.
[15] C.C. Kiehlbauch, N. Aboul-Ela, E.L. Jacobson, D.P. Ringer and M.K. Jacobson, *Anal. Biochem.*, 208 (1993) 26.
[16] L.A. Loeb, *J. Biol. Chem.*, 244 (1969) 1672.
[17] P.L. Panzeter and F.R. Althaus, *Nucl. Acids Res.*, 18 (1990) 2194.
[18] J. Ménissier-de Murcia, M. Molinete, G. Gradwohl, F. Simonin and G. de Murcia, *J. Mol. Biol.*, 210 (1989) 229.
[19] H. Naegeli and F.R. Althaus, *J. Biol. Chem.*, 266 (1991) 10596.
[20] U.K. Laemmli, *Nature*, 227 (1970) 680.