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Purification of a Low-Molecular-Weight Phospholipase A_2 Associated with Soluble High-Molecular-Weight Acidic Proteins from Rabbit Nucleus Pulposus and Its Comparison with a Rabbit Splenic Group IIa Phospholipase A_2[1]

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An intervertebral disc is a large peice of avascular cartilage rich in proteoglycans and water consisting of gelatinous nucleus pulposus and fibrous annulus fibrosus. The soluble fraction of rabbit nucleus pulposus exhibited unusually high Ca^{2+}-dependent phospholipase A_2 (PLA_2) activity (about 70% of the total PLA_2 activity). The soluble PLA_2 activity was 6–7-fold higher than those of rabbit annulus fibrosus and spleen. The PLA_2 was bound to an anion-exchange column at pH 7.4, and eluted near the void volume as a broad peak on gel-filtration on a TSKgel SuperSW3000 column developed with a buffer containing 0.1–0.2 M salt. When the gel-filtration column was developed in the presence of 1 M salt, almost all the PLA_2 activity was eluted near the total available volume. The soluble PLA_2 was purified to near homogeneity. A Ca^{2+}-dependent PLA_2 was also purified from the fractions extracted with 1 M KBr from nucleus pulposus. For comparison, we purified a Ca^{2+}-dependent PLA_2 from the KBr fraction of spleen. The splenic PLA_2 was identical to a group IIa PLA_2, as judged from its N-terminal amino acid sequences and mass spectra. On SDS–polyacrylamide gel electrophoresis the enzymes purified from the soluble and KBr fractions of nucleus pulposus both gave a major 15.7-kDa band at the same position as splenic group IIa PLA_2. These results suggest that group IIa PLA_2 is associated with soluble high-molecular-weight proteins, most likely proteoglycans, in the extracellular matrix of rabbit nucleus pulposus.

Key words: chondrocyte, intervertebral disc, nucleus pulposus, phospholipase A_2, proteoglycan.

An intervertebral disc is a large piece of avascular cartilage rich in proteoglycans and water (70–80% of the tissue weight is due to water; for a review, see Ref. *1*). It consists of nucleus pulposus and annulus fibrosus, the latter laterally surrounding the former to prevent it from slipping out of the proper place. The disc is connected to neighboring vertebra through end plates, which are thin pieces of hyaline cartilage. Discs allow the spine not only to resist huge axial loads but also to move in various directions. Cartilage cells are the sole cells sparsely found in the large avascular extracellular matrix. Because loss of the integrity of a disc causes low back pain and degenerative disc diseases such as disc herniation, it is important to understand how the cells keep the extracellular matrix intact.

PLA_2s hydrolyze the *sn*-2 fatty acid acyl ester bonds of phosphoglycerides to yield free fatty acids and lysophospholipids, and have various physiological functions (for reviews, see Refs. *2–4*). In mammals, four nonpancreatic low-molecular-weight (about 14-kDa) PLA_2s are now known (*5*). These secretory PLA_2s (groups IIa, V, IIc, and X) are each expressed in a tissue-specific manner, and all of them require mM-levels of Ca^{2+} to be fully active and show maximal activity in the pH range of 7–9. The physiological functions of these secretory PLA_2s have been vigorously investigated, but remain unclear in many aspects.

In the previous study (*6*), we biochemically demonstrated that normal human discs contain high levels of group IIa PLA_2, and that in diseased discs the amounts of the enzyme decrease. The cause of PLA_2 accumulation at high levels and the source of the enzyme are still controversial problems (*7*, *8*), although our results strongly suggest that group IIa PLA_2 plays some important physiological roles in

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Abbreviations: ADAM, 9-anthryldiazomethane; PLA_2, phospholipase A_2; PLA_2-S, phospholipase A_2 in the soluble fraction of nucleus pulposus and purified from the Super Q-Toyopearl-bound enzyme fraction concentrated by ultrafiltration with a CF25 membrane cone; PLA_2-SF, phospholipase A_2 in the soluble fraction of nucleus pulposus and purified from the 1 M KCl filtrate of the Super Q-Toyopearl-bound enzyme fraction by ultrafiltration with a CF25 membrane cone; PLA_2-M, phospholipase A_2 purified from the KBr fraction of nucleus pulposus, possibly a membrane-bound enzyme; POPG, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoglycerol; V_0, void volume; V_t, total available volume.

ordinary discal metabolism. Recently, it was reported that human group IIa PLA_2 binds to glycosaminoglycans from proteoglycans secreted by cultured human arterial smooth muscle cells, and that glycosaminoglycans modulate the activity of the enzyme in a dose-dependent manner (*9*). The basic amino acid residues responsible for the binding of the PLA_2 to glycosaminoglycans have been determined (*10*, *11*). The extracellular matrix of intervertebral discs comprises a proteoglycan-water gel strengthened by collagen fibrils and hyaluronate, and most of the proteoglycans are easily extracted from the matrix with water or dilute salts (*12*, *13*). In previous studies including ours (*6*, *14*, *15*), human disc materials were homogenized in a buffer containing 1–1.6 M salt to determine the total Ca^{2+}-dependent PLA_2 activity. Thus, it is important to examine the differential distribution of PLA_2 activity in the soluble and salt-extractable fractions of discs.

In this study, we examined the distribution of Ca^{2+}-dependent PLA_2 activity in both the soluble and 1 M KBr-extractable fractions of rabbit nucleus pulposus, annulus fibrosus, and spleen. In the case of rabbit nucleus pulposus, the soluble fraction contained higher PLA_2 activity than the KBr fraction. We purified, to near homogeneity, both the PLA_2 in the supernatant fraction of nucleus pulposus and that in the KBr fraction. We also purified to homogeneity a group IIa PLA_2 from the KBr fraction of rabbit spleen. We obtained the first evidence that a low-molecular-weight PLA_2 is associated with soluble high-molecular-weight proteins in the extracellular matrix of nucleus pulposus.

MATERIALS AND METHODS

Materials—Japanese white rabbits (3–4 kg) anesthetized with pentobarbital were sacrificed by drawing blood from the cervical artery, and then the spleen and vertebral column were removed en bloc from each rabbit. From each lumbar and thoracic disc, the nucleus pulposus was first removed with a microspatula, and then the annulus fibrosus was excised from the remaining disc material. The samples were immediately frozen and stored at –20°C.

Assay for PLA_2 Activity—We separately determined PLA_2 activity in disc and spleen samples obtained from five rabbits.

Each spleen sample (1–1.6 g) was homogenized in 10 volume of 10 mM Tris-HCl (pH 7.4) on ice with a glass homogenizer. After 20-min incubation in ice-water, the homogenate was centrifuged at 130,000 ×*g* for 20 min at 4°C. An aliquot (1 μl) of the supernatant (soluble fraction) was used for the assay. The pellet was homogenized in the same volume of 10 mM Tris-HCl (pH 7.4) containing 1 M KBr on ice with a glass homogenizer. After 20-min incubation in ice-water, the homogenate was centrifuged at 130,000 ×*g* for 20 min at 4°C. A 1-μl aliquot of the supernatant (KBr fraction) was used for the assay.

Each nucleus pulposus (0.1–0.18 g) or annulus fibrosus sample (0.5–0.7 g) was first crushed into a fine powder, in a stainless steal container cooled with liquid nitrogen, with a cryopress (CP-100W; Microteque Nition). Then, soluble and KBr fractions were prepared from the powdered samples as described for the spleen samples.

PLA_2 activity was determined by a HPLC method based on precolumn derivatization of fatty acids with 9-anthryldiazomethane (ADAM) (*16*) with slight modifications. To minimize the disturbance on the detection of derivatized fatty acids by peaks derived from commercial ADAM reagents (Funakoshi, Tokyo), the fluorescence of the effluent was monitored at 412 nm, with excitation at 365 nm, by means of a Shimadzu RF-535 fluorescence detector. ADAM-labeled fatty acids (oleic acid, palmitic acid, and heptadecanoic acid) were rapidly separated on a Superspher RP-18 column (4 × 50 mm) at 22°C and the flow rate of 1.5 ml/min (8 min needed for one analysis). The assay mixture (50 μl) contained 0.8 mM 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoglycerol (POPG) (Avanti Polar Lipids), 5 mM cholate, 0.1 M Tris-HCl (pH 8.5), 0.1 M NaCl, 5 mM $CaCl_2$, and the sample solution. Heptadecanoic acid (Nacalai Tesque, Tokyo) was used as an internal standard. Calcium-dependent PLA_2 activity was estimated as the difference between the activity detected in the presence of 5 mM $CaCl_2$ and that in the presence of 10 mM EDTA.

Purification of Splenic PLA_2—PLA_2 was purified from the KBr fraction of rabbit spleen (56 g wet weight) by chromatography on a SP Sepharose column (5 × 15 cm, Pharmacia Biotech), ultrafiltration with a Centriflo CF25 membrane cone (Amicon), chromatography on a SP Sepharose column (1 × 1.2 cm), and chromatography on a Cosmosil 5C4-300 AR column (2.0 × 150 mm, Nacalai Tesque), as described previously for the purification of group IIa PLA_2 from human intervertebral discs (*6*), with the following modifications. In contrast to human disc PLA_2, most PLA_2 activities in rabbit spleen were recovered from the filtrates, and then directly applied to the C4 column after concentration with the short SP-Sepharose column. For purification of the PLA_2, the ultrafiltration step with a Centriflo CF25 membrane cone was as effective as HPLC gel filtration.

We obtained 35 μg of the pure enzyme with specific activity of 245 μmol/min/mg. The purified enzyme migrated as a single band corresponding to an apparent molecular mass of 15.7 kDa (Fig. 4). The purified splenic PLA_2 was confirmed to be a rabbit group IIa PLA_2 (*17–20*) by its mass spectrum and NH_2-terminal amino acid sequence.

Purification of Ca^{2+}-Dependent PLA_2 from Rabbit Nucleus Pulposus—Rabbit nucleus pulposus (2.4 g) was homogenized in 40 ml of 10 mM Tris-HCl (pH 7.4). After 10-min incubation on ice, the homogenate was centrifuged at 130,000 ×*g* for 20 min at 4°C. The supernatant (soluble fraction) was applied to a Super Q-Toyopearl column (2 × 14 cm; Tosoh) preequilibrated with 10 mM Tris-HCl (pH 7.4). The column was washed with 400 ml of 25 mM Tris-HCl (pH 7.4) containing 0.1 M KCl, and then the PLA_2 activity bound to the column was eluted with 25 mM Tris-HCl (pH 7.4) containing 1 M KCl and 0.1% Triton X-100. The pooled PLA_2-active fractions (Super Q-Toyopearl) were concentrated to 1.8 ml by ultrafiltration with a Centriflo CF25 membrane cone. The concentrate was 10-fold diluted with 25 mM Tris-HCl (pH 7.4) containing 1 M KCl and 0.1% Triton X-100, and then concentrated again by ultrafiltration with the same membrane. This dilution-concentration process was repeated one more time. From the combined filtrate, 20% of the PLA_2 activity was recovered and then concentrated to 0.25 ml with a Microcon YM-3 membrane filter (Amicon); this PLA_2 preparation is designated as PLA_2-SF. The main PLA_2 activity recovered from the final concentrate was further purified by gel-filtration on a TSKgel $G3000SW_{XL}$ column (7.8 × 300 mm; Tosoh). Aliquots (100 μl) of the PLA_2 sample were repeatedly injected

onto the column preequilibrated with 50 mM HEPES (pH 7.4) containing 1 M KCl and 0.1% Triton X-100. Elution was performed with the same buffer at the flow rate of 0.4 ml/min. The pooled PLA_2-active fractions were concentrated to 0.24 ml (TSKgel G3000SW) by ultrafiltration with a Microcon YM-3 membrane filter; this PLA_2 preparation is designated as PLA_2-S.

The precipitate obtained from the crude nucleus pulposus homogenate on centrifugation was homogenized in 40 ml of 10 mM Tris-HCl (pH 7.4) containing 1 M KBr, and then centrifuged at 130,000 $\times g$ for 20 min. The supernatant (KBr extract) was diluted 10-fold with cold water, and then applied to a SP-Toyopearl column (2.5 × 2 cm; Tosoh) preequilibrated with 10 mM Tris-HCl (pH 7.4). The column was washed with 10 mM Tris-HCl (pH 7.4) containing 0.1 M KCl and 0.1% Triton X-100. The PLA_2 activity bound to the column was eluted with 10 mM Tris-HCl (pH 7.4) containing 1 M KCl and 0.1% Triton X-100. The pooled PLA_2-active fractions (SP-Toyopearl) were concentrated to 0.25 ml by ultrafiltration with a Microcon YM-3 filter, and then further purified by gel-filtration on a TSKgel SuperSW3000 column (4.6 × 300 mm; Tosoh). Aliquots (30 μl) of the PLA_2 sample were repeatedly injected onto the column preequilibrated with 50 mM HEPES (pH 7.4) containing 0.2 M KCl and 0.1% Triton X-100. Elution was performed with the same buffer at the flow rate of 0.2 ml/min. The pooled PLA_2-active fractions were concentrated to 0.15 ml (TSKgel SuperSW) by ultrafiltration with a YM-3 membrane filter; this PLA_2 preparation is designated as PLA_2-M.

SDS–Polyacrylamide Gel Electrophoresis—SDS-PAGE was performed on a 15% gel according to Laemmli (*21*) under reducing conditions. Aliquots (2.0 μl) of PLA_2-S, PLA_2-SF, and PLA_2-M were 11-fold diluted with 25 mM Tris-HCl (pH 7.4) containing 0.1% Triton X-100. An aliquot of the splenic PLA_2 was diluted 100-fold with the same buffer. We determined PLA_2 activity for all the diluted enzyme solutions, and each of them containing the same amount of PLA_2 activity (2.4 nmol/min, 7–12.5 μl) was mixed with 7.5 μl of Laemmli's sample buffer, incubated at 80°C for 4 min, and then electrophoresed in a 15% SDS-PAGE gel. Proteins were stained with a silver staining kit (Wako Pure Chemical Industries).

To determine the NH_2-terminal amino acid sequences of the PLA_2s, aliquots (1 μg, 14–24 μl) of PLA_2-S, PLA_2-SF, and PLA_2-M were diluted with 400 μl of distilled water containing 15 μg of bovine serum albumin (crystalline; Sigma), concentrated to about 10 μl by ultrafiltration with a YM-3 membrane filter, and then electrophoresed in a 15% SDS-PAGE gel after treatment with 7.5 μl of Laemmli's sample buffer. The separated proteins were electro-transferred to a PVDF membrane (Clear Blot Membrane-P; ATTO) with a semi-dry blotter (AE-6677-S; ATTO), and then stained with Coomassie Brilliant Blue. The membrane was rinsed 3 times with 50% methanol. The protein bands corresponding to 15.6 kDa were excised, and each of the membrane slices was directly examined with an Applied Biosystems 477A sequencer and a 120A PTH analyzer. The marker proteins were purchased from Boehringer Mannheim.

Other Analytical Methods—The protein concentrations of crude samples were determined in a final reaction volume of 420 μl with a bicinchoninic acid protein assay kit (Pierce), and those of the purified enzyme were estimated in a final reaction volume of 120 μl with the kit using a Shimadzu UV-2200 spectrophotometer equipped with an ultra micro cell holder. The amino acid sequences of the purified splenic PLA_2 were determined with an Applied Biosystems 470A sequencer and a 120A PTH analyzer. Matrix-assisted laser desorption mass spectra were obtained with a Voyager-DE RP mass spectrometer (PerSeptive Biosystems). α-Cyano-4-hydroxycinnamic acid was used as a matrix-forming material. Apomyoglobin and thioredoxin were used for mass calibration.

RESULTS

Distribution of Ca^{2+}-Dependent PLA_2 Activity in Rabbit Nucleus Pulposus, Annulus Fibrosus, and Spleen—We prepared soluble and KBr fractions from rabbit nucleus pulposus, annulus fibrosus and spleen as described under "MATERIALS AND METHODS," and determined the Ca^{2+}-dependent PLA_2 activity in each fraction. Figure 1 shows the results. The spleen is one of the rich sources of group IIa PLA_2 (*22*, *23*), and so we examined rabbit splenic PLA_2 activity for comparison. The total PLA_2 activity of nucleus pulposus and annulus fibrosus was about 50 and 20% of the total splenic activity, respectively (Fig. 1A). Surprisingly, the PLA_2 activity in the soluble fraction of nucleus pulposus was 7-fold higher than that of spleen. The PLA_2 activity in the soluble fraction of annulus fibrosus was comparable to that of spleen. The ratio of the PLA_2 activity in the supernatant fraction to that in the KBr fraction was 2.5 for

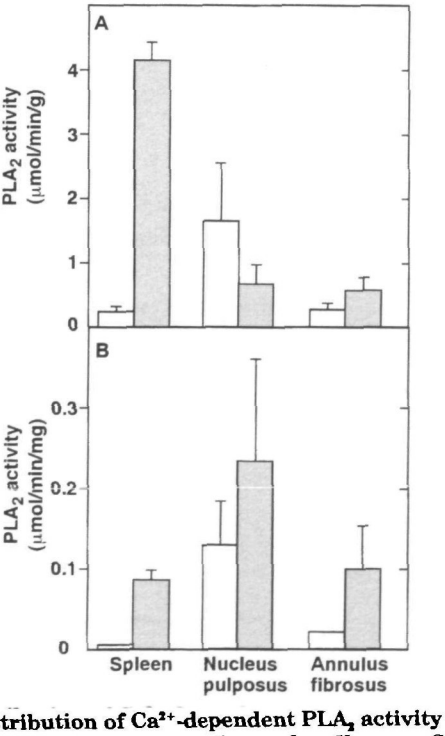

Fig. 1. **Distribution of Ca^{2+}-dependent PLA_2 activity in rabbit spleen, nucleus pulposus, and annulus fibrosus.** Ca^{2+}-dependent PLA_2 activity in the soluble (open columns) and KBr fractions (closed columns) of spleen, nucleus pulposus, and annulus fibrosus was determined. Values are means ± SD for five rabbits. PLA_2 activity was measured as described under "MATERIALS AND METHODS" using 0.8 mM POPG plus 5 mM cholate mixed micelles. The enzyme activities are compared in terms of the activity per g tissue (A) and specific activity (B).

nucleus pulposus, 0.5 for annulus fibrosus, and 0.06 for spleen.

As shown in Fig. 1B, the specific activity in the soluble fraction of nucleus pulposus was 0.13 ± 0.05 μmol/min/mg, this value being 6- and 30-fold greater than those of annulus fibrosus and spleen, respectively. The specific activity of the KBr fraction of nucleus pulposus was 0.23 ± 0.13 μmol/min/mg, *i.e.* about 2-fold higher than those of annulus fibrosus and spleen.

Purification of Ca^{2+}-Dependent PLA_2 in the Soluble Fraction of Nucleus Pulposus—We purified to near homogeneity the Ca^{2+}-dependent PLA_2 in the soluble fraction of nucleus pulposus. The results of the purification are summarized in Table I. A part of the enzyme activity (about 30%) was recovered in the flow-through fraction on extensive washing (10 column volumes) of a Super Q-Toyopearl column with 10 mM Tris-HCl (pH 7.4) containing 0.1 M KCl, and the remaining activity bound to the column was eluted with 10 mM Tris-HCl (pH 7.4) containing 1 M KCl and 0.1% Triton X-100 (Fig. 2A). We purified Ca^{2+}-dependent PLA_2 from the latter fraction because of its acidic nature and higher specific activity. The pooled enzyme fractions were concentrated to about 1.8 ml by ultrafiltration with a Centriflo CF25 membrane cone. To remove low molecular weight proteins, the concentrate was washed twice with 10 mM Tris-HCl (pH 7.4) containing 1 M KCl and 0.1% Triton X-100 by repeated 10-fold dilution with the buffer and re-concentration by ultrafiltration. About 20% of the PLA_2 activity was recovered from the pooled filtrate, and we concentrated the filtrate to 0.25 ml by ultrafiltration with a Microcon YM-3 membrane filter. The PLA_2 (PLA_2-SF, 18 μg) showed specific activity of 25.1.

The main PLA_2 activity was recovered in the concentrate obtained on ultrafiltration, and it was further purified by gel-filtration. Figure 2B shows the elution profiles of PLA_2 activity and protein on a TSKgel SuperSW3000 column (4.6 × 300) developed with 50 mM HEPES (pH 7.4) containing 0.2 M NaCl and 0.1% Triton X-100. Most PLA_2 activity was eluted near the void volume (V_0) as a broad peak, and a substantial amount of the activity was eluted at the total available volume (V_t). This result suggested that a low molecular weight PLA_2 interacts with high molecular weight proteins, and that the interaction was partially disrupted by the presence of 0.2 M NaCl. To confirm this pos-

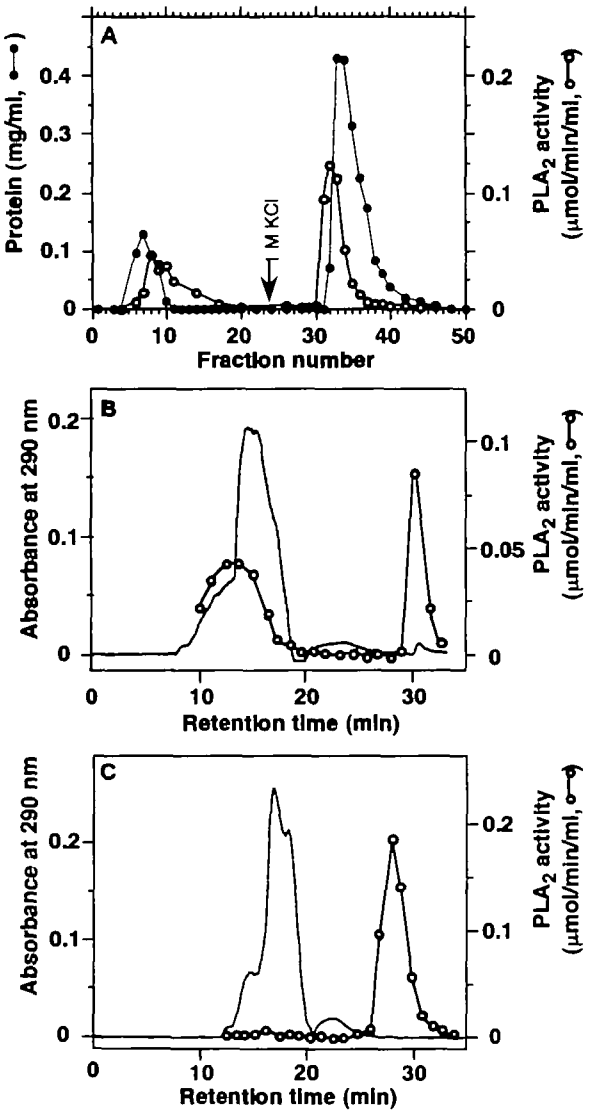

Fig. 2. **Chromatographic profiles of protein and Ca^{2+}-dependent PLA_2 activities in the soluble fraction of rabbit nucleus pulposus on a Super Q Toyopearl column (A), a TSKgel SuperSW3000 column (B), and a TSKgel G3000SW_{XL} column (C).** PLA_2 activity was measured as described under "MATERIALS AND METHODS" using 0.8 mM POPG plus 5 mM cholate mixed micelles. The reaction volume was 50 and 2 μl of each fraction was used for the enzyme assay. (A) The soluble fraction (35 ml) was prepared and applied to an anion-exchange column as described under "MATERIALS AND METHODS." The column was washed with 25 mM Tris-HCl (pH 7.4) containing 0.1 M KCl, and then the bound proteins were eluted with the buffer containing 1 M KCl and 0.1% Triton X-100. (B) PLA_2-active fractions eluted with 1 M KCl from the Super Q column (see Fig. 2A) were pooled, and concentrated by ultrafiltration as described under "MATERIALS AND METHODS," and then an aliquot of the concentrate (30 μl) was applied to a gel-filtration column. The column was developed at the flow rate of 0.2 ml/min, and 0.2 ml fractions were collected. The eluent contained 0.2 M NaCl and 0.1% Triton X-100. Catechol 2,3-dioxygenase (M_r of 140,000), bovine serum albumin monomer (M_r of 66,000), and KCl (M_r of 74.6) were eluted at 19.2, 20.5, and 29.2 min, respectively. (C) The same sample as that applied to the TSKgel SuperSW3000 column was subjected to gel-filtration on a TSKgel G3000SW_{XL} column. The sample volume applied was 100 μl, and 0.4 ml fractions were collected. The column was developed at the flow rate of 0.4 ml/min, and the eluent contained 1 M KCl and 0.1% Triton X-100.

TABLE I. **Purification of a Ca^{2+}-dependent PLA_2 in the soluble fraction of nucleus pulposus from rabbit intervertebral discs.**

Purification step	Total volume (ml)	Total activity (μmol/min)	Total protein (mg)	Specific activity (μmol/min/mg)	Yield (%)
Soluble fraction	35.0	4.38	36.4	0.120	100
Super Q-Toyopearl	34.5	2.49	5.6	0.443	56.8
TSKgel G3000SW	0.24	0.62	0.010	61.0	14.2

sible interaction, we applied an aliquot of the PLA_2 sample to a TSKgel $G3000SW_{XL}$ column, and developed the column with 50 mM HEPES (pH 7.4) containing 1 M KCl and 0.1% Triton X-100 (Fig. 2C). Almost all the PLA_2 activity was eluted at the elution volume nearly corresponding to V_t of the column. This gel-filtration step in the presence of 1 M KCl was very effective for purification, leading to 140-fold purification through this single step. The pooled PLA_2 was concentrated to 0.24 ml by ultrafiltration (PLA_2-S). PLA_2-S (10 μg) showed specific activity of 61.0 μmol/min/mg.

Purification of Ca^{2+}-Dependent PLA_2 in the KBr Fraction of Nucleus Pulposus—We purified to near homogeneity the Ca^{2+}-dependent PLA_2 in the KBr fraction of nucleus pulposus. The results of the purification are summarized in Table II. Nearly all the PLA_2 activity was bound to a SP-Toyopearl column, and eluted as a relatively broad peak with 10 mM Tris-HCl (pH 7.4) containing 1 M KCl and 0.1% Triton X-100 (Fig. 3A). The pooled enzyme fractions were concentrated by ultrafiltration, and further purified by gel-filtration. Figure 3B shows the elution profiles of PLA_2 activity and protein on a TSKgel SuperSW3000 column (4.6 × 300) developed with 50 mM HEPES (pH 7.4) containing 0.2 M NaCl and 0.1% Triton X-100. The PLA_2 activity was eluted at the total available volume. The pooled PLA_2 was concentrated to 0.15 ml by ultrafiltration (PLA_2-M). PLA_2-M (8 μg) showed specific activity of 38.1 μmol/min/mg.

Confirmation of the Purified Splenic PLA_2—The molecular mass of the purified splenic PLA_2 was determined to be 14,099 Da by mass spectrometry (the calculated value for a rabbit group IIa PLA_2 is 14,091). The NH_2-terminal amino acid sequence of the splenic PLA_2 was determined to be HLLDFRKMIRYTTGKEATTSYGAYG?H?GVGGRG. These results confirmed that the purified splenic PLA_2 was a rabbit group IIa PLA_2 (*17–20*).

SDS-polyacrylamide Gel Electrophoresis and NH_2-Terminal Sequencing—The enzymes purified from nucleus pulposus (PLA_2-S, PLA_2-SF, and PLA_2-M), and the splenic PLA_2 were analyzed by SDS-PAGE (Fig. 4A). All the enzyme solutions subjected to SDS-PAGE contained an equal amount of enzyme activity (2.4 nmol/min). Both PLA_2-S and PLA_2-SF gave a major band corrsponding to an apparent M_r of 15,700 at the same position to which the splenic group IIa PLA_2 migrated. Compared to the splenic PLA_2, PLA_2-S and PLA_2-SF gave slightly stronger 15.6 kDa protein bands. PLA_2-M gave a much stronger 15.6 kDa band than the other three PLA_2s, although all four enzyme preparations contained the same amount of enzyme activity.

To determine the NH_2-terminal amino acid sequences of PLA_2-S, PLA_2-SF, and PLA_2-M, the enzymes (1.0 μg each) were desalted by ultrafiltration in the presence of bovine serum albumin, and then electrophoresed in a 15% SDS-PAGE gel, blotted onto a PVDF membrane, and stained with Coomassie Brilliant Blue (Fig. 4B). A 15.6 kDa band was clearly observed for PLA_2-M, weakly observed for PLA_2-SF, and faintly observed for PLA_2-S (too faint to be visible in the figure). Figure 4C shows the first five chromatograms obtained on NH_2-terminal sequencing of PLA_2-S, PLA_2-SF, and PLA_2-M. The NH_2-terminal amino acid sequences of PLA_2-S, PLA_2-SF, and PLA_2-M were determined to be HLLDFRKMIR?TTGKEATT, HLLDFRKMIR, and HLLDFRKMIR, respectively. Based on these results, PLA_2-S, PLA_2-SF, and PLA_2-M can be assigned as a rabbit group IIa PLA_2. The significant difference in the recovery observed among PLA_2-S, PLA_2-SF, and PLA_2-M (Fig. 4, A and

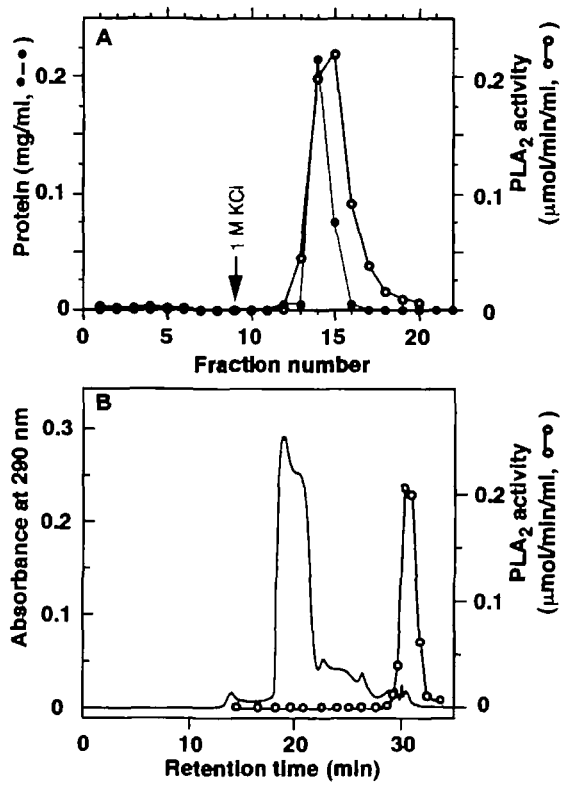

Fig. 3. **Chromatographic profiles of protein and Ca^{2+}-dependent PLA_2 activities in the KBr fraction of rabbit nucleus pulposus on a SP Toyopearl column (A) and a TSKgel SuperSW3000 column (B).** PLA_2 activity was measured as described under "MATERIALS AND METHODS" using 0.8 mM POPG plus 5 mM cholate mixed micelles. The reaction volume was 50 μl and 2 μl of each fraction was used for the enzyme assay. (A) The KBr fraction (38 ml) was diluted 10-fold with cold water, and then applied to a cation-exchange column as described under "MATERIALS AND METHODS." The column was washed with 10 mM Tris-HCl (pH 7.4) containing 0.1 M KCl and 0.1% Triton X-100, and then the bound proteins were eluted with the buffer containing 1 M KCl and 0.1% Triton X-100. (B) PLA_2-active fractions eluted with 1 M KCl from the SP Toyopearl column (see Fig. 3A) were pooled, and concentrated by ultrafiltration as described under "MATERIALS AND METHODS." An aliquot of the concentrate (30 μl) was applied to a gel-filtration column. The column was developed at the flow rate of 0.2 ml/min, and 0.2 ml fractions were collected. The eluent contained 0.2 M NaCl and 0.1% Triton X-100.

TABLE II. **Purification of a Ca^{2+}-dependent PLA_2 in the KBr fraction from rabbit nucleus pulposus.**

Purification step	Total volume (ml)	Total activity (μmol/min)	Total protein (mg)	Specific activity (μmol/min/mg)	Yield (%)
KBr extract	38.0	1.77	9.84	0.180	100
SP-Toyopearl	12.5	0.83	1.0	0.826	46.6
TSKgel SuperSW	0.15	0.29	0.008	38.1	16.3

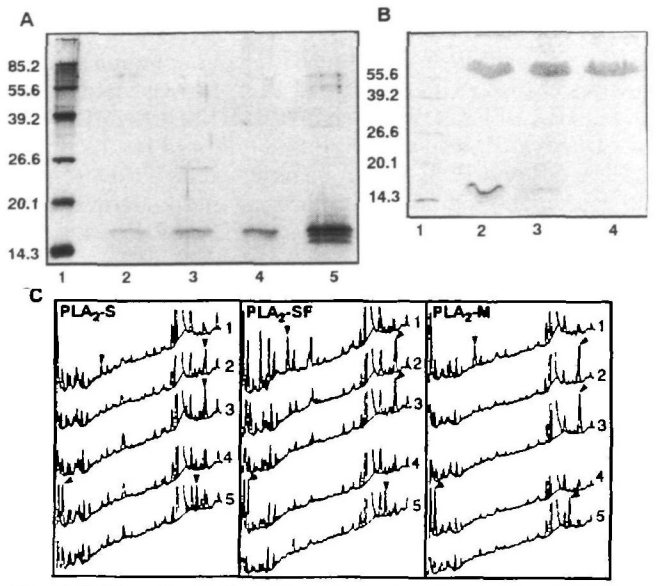

Fig. 4. **SDS–polyacrylamide gel electrophoresis and NH_2-terminal sequencing of the soluble and KBr-extracted PLA_2s from rabbit nucleus pulposus.** (A) Aliquots of splenic IIa PLA_2 (lane 2), PLA_2-S (lane 3), PLA_2-SF (lane 4), and PLA_2-M (lane 5), each containing 2.4 nmol/min PLA_2 activity, were electrophoresed in a 15% gel as described under "MATERIALS AND METHODS." Lane 1 shows the marker proteins; the values are the respective molecular mass expressed in kDa. Proteins were stained with silver. (B) Aliquots (1.0 μg) of PLA_2-M (lane 2), PLA_2-SF (lane 3), and PLA_2-S (lane 4) were desalted in the presence of 15 μg of bovine serum albumin by dilution with water and subsequent ultrafiltration, and then subjected to electrophoresis on a 15% gel. The proteins on the gel were electro-transferred onto a PVDF membrane and then stained with Coomassie Brilliant Blue. The strong bands that migrated to just above the 55.6 kDa marker band are bovine serum albumin, which was used as a carrier to prevent the PLA_2s from being lost during the sample treatment. Lane 1 shows the marker proteins. PLA_2-S (lane 4) is too faintly stained to be visible in the figure. (C) Each 15.7 kDa band on the membrane (Fig. 4B) was excised and the membrane slice was directly examined with a protein sequencing system. The first five chromatograms are shown. The arrowheads indicate His (residue 1), Leu (residues 2 and 3), Asp (residue 4), and Phe (residue 5), respectively.

B) is, at least partly, due to nonspecific adsorption of the enzyme to the plastics used for sample treatment because PLA_2-M contained significant amounts of contaminating proteins with low-molecular weights, as judged on SDS-PAGE, and showed the best recovery.

DISCUSSION

In this study we found the dominant distribution of Ca^{2+}-dependent PLA_2 activity in the soluble fraction of rabbit nucleus pulposus. About 70% of the total enzyme activity was in the soluble fraction. In contrast, only 5% of the total PLA_2 activity of rabbit spleen was found in the soluble fraction. Like rabbit spleen, in mammalian tissues examined hitherto, Ca^{2+}-dependent PLA_2 activity has been found mostly in fractions solubilized with strong acids, high concentrations of salts, or detergents, with only a small fraction of the activity in the soluble fraction (*22–24*).

Rabbit splenic PLA_2 was purified to homogeneity from the KBr fraction and unambiguously identified as a group IIa PLA_2. The PLA_2 in the KBr fraction of nucleus pulposus exhibited properties quite similar to those of the spleen enzyme in each purification step, suggesting that the two enzymes are identical.

Unlike these PLA_2s, the crude PLA_2 in the soluble fraction of nucleus pulposus did not bind to cation-exchange columns such as SP-Toyopearl and Mono S ones (data not shown). An unusually large amount of Super Q-Toyopearl gel (44 ml), an amount enough to bind 5–6 g of bovine serum albumin, was necessary to retain most of the PLA_2 activity in the crude soluble fraction, which contained only 36.4 mg of protein. When gel-filtration on a TSKgel $G3000SW_{XL}$ column was carried out under dissociative conditions in the presence of 1 M KCl, almost all the PLA_2 activity was eluted at the total available volume, whereas most PLA_2 activity was eluted near the void volume under weakly dissociative conditions. Taking these results together, it is strongly suggested that a low molecular weight PLA_2 interacts with high-molecular-weight polyanionic proteins, probably proteoglycans.

The Ca^{2+}-dependent PLA_2 activity in the soluble fraction of rabbit nucleus pulposus was 6-fold higher than that of annulus fibrosus, whereas the activity in the KBr fraction of nucleus pulposus and that of annulus fibrosus were the same. The soluble fraction of human nucleus pulposus contained 2–4 times higher levels of proteoglycans than that of human annulus fibrosus (*12*). About 70% of the proteoglycans in human discs are recovered in the soluble fraction, and the yield does not change with an increase in the salt concentration in the extractant used in the range of 0–4 M (*13*). Based on these results obtained for human discs, the difference in the levels of Ca^{2+}-dependent PLA_2 activity in the soluble fractions of rabbit nucleus pulposus and annulus fibrosus is possibly due to the difference in the levels of soluble proteoglycans in nucleus pulposus and annulus fibrosus. In terms of specific activity, the difference in the levels of PLA_2 activity in the KBr fractions of spleen, nucleus pulposus, and annulus fibrosus is very small compared to that in the soluble fraction. This suggests that PLA_2 activity in the KBr fraction is mainly due to membrane-associated or cell-associated PLA_2.

Human intervertebral discs contain high levels of group IIa PLA_2 (*6*, *14*, *15*). The source of the enzyme, the localization of the enzyme, the cause of high levels of accumulation of the enzyme, and the physiological roles of the enzyme in discs are all still unclear. The present study suggests that the disc extracellular matrix acts as a reservoir with a great capacity for PLA_2. Confirmation of the localization of the enzyme in the extracellular matrix by immunohistochemistry is predicted to be difficult because most proteoglycans, to which PLA_2 is bound, are soluble and can be easily lost upon sample treatment for staining. Recently, it was found that the potent anti-Gram-positive bacteria activity of a rabbit inflammatory fluid is due to a group IIa PLA_2 (*18*). The extracellular PLA_2 concentration of rabbit nucleus pulposus is estimated to be about 470 nM, using the specific activity of the rabbit splenic group IIa PLA_2 purified in this study and the molecular weight of 14,091. This concentration is about 50-times higher than the LD_{50} toward *Staphylococcus aureus*. These findings suggest that the PLA_2 is an endogenous antibiotic in a large avascular intervertebral disc.

Low back and radicular pain induced by lumbar disc her-

niation is not always due to mechanical compression of the nerve root, but more often due to a local chemical contribution (*25*). Injection or allografting of nucleus pulposus into the lumbar epidural space produces an inflammatory response to the nuclear material injected and mechanical hyperalgesia (*25*, *26*). Mammalian cells are variously activated when they are exposed to high doses of extracellular group IIa PLA_2, and the intact enzymatic activity is not always necessary for the PLA_2 to activate target cells (*1–4*, *27*). Because rabbit nucleus pulposus contains high levels of extracellular PLA_2 associated with its extracellular matrix components with moderate strength, it is important to determine whether or not the PLA_2 in nucleus pulposus induces low back and radicular pain as a local chemical stimulant which stays at a local position for a long time.

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