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Determination of profiles of non-collagenous proteins from rat bones by sodium dodecyl sulfate high-performance liquid chromatography

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ABSTRACT

Sodium dodecyl sulfate–high-performance liquid chromatographic (SDS-HPLC) techniques for screening profiles of bone non-collagenous proteins (NCP) are described and compared with sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) techniques. NCPs were obtained from long bones of neonatal and mature rats by sequential extraction with 4 *M* guanidine hydrochloride (GdnHCl) and 0.25 *M* EDTA followed by desalting. Desalted extracts were subjected to SDS-PAGE and SDS-HPLC. The results of the two analyses were comparable. There were differences in NCP profiles between mature rats and pups and between the GdnHCl and EDTA extracts. The methods described can be used for qualitative comparison of treatments and as a basis for further study.

INTRODUCTION

The analysis of individual non-collagenous proteins (NCPs) from hard tissues has traditionally been accomplished by harsh extraction steps followed by multiple liquid chromatographic, precipitation and concentration procedures [1,2]. Among the methods that have been developed to characterize the molecular weights of all the NCPs from rat compact bone are gel filtration chromatography followed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) [3], fractional precipitations and ion-exchange chromatography followed by SDS-PAGE [3], calcium-induced precipitation followed by high-performance liquid chromatography (HPLC) [4] and two-dimentional gel electrophoresis with electroblotting [5]. The development of a rapid method that allows the comparison of NCPs *in toto* would be a useful screening tool for studies of the effects of nutritional stresses and disease states on the NCP composition of bone. When differences are identified by these comparisons, further studies on those specific NCPs can be undertaken.

EXPERIMENTAL

Materials

Bones were obtained from crl:COBS Sprague–Dawley descended rats (Charles Rivers Labs., Research Triangle Park, NC, USA). Reagents used for extractions were of either analytical-reagent or electrophoresis grade and were obtained from Fisher (Norcross, GA, USA)) or Sigma (St. Louis, MO, USA). Spectra Por 3 dialysis tubing (molecular weight cut-off 3500) was purchased from Spectrum Medical Industries (Los Angeles, CA, USA). Sephadex G-25-150 and PD-10 columns were obtained from Pharmacia (Uppsala, Sweden). All reagents and equipment used for electrophoresis and silver staining and the low-molecular-weight standards were supplied by Bio-Rad Labs. (Richmond, CA, USA). The HPLC equipment was obtained from Waters (Milford, MA, USA) and all reagents used for HPLC were of HPLC grade.

Sample preparation and NCP extraction

Femurs and tibias were removed from newborn rat pups and their dams and cleaned of soft tissues

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and marrow. The midshafts were isolated and placed in a cold solution of 4 *M* guanidie hydrochloride (GdnHCl) with a "cocktail" of protease inhibitors [2]. The solution containing the bones from the pups was placed on a rotary shaker at 4°C for 96 h. The bones from the dams were removed from he GdnHCl solution, lyophilized, crushed to increase the surface area and subjected to the same extraction procedure as the bones from the pups.

After 96 h, the GdnHCl solution was decanted and the bones were covered with 0.25 *M* EDTA–protease inhibitors solution [2] and placed on a rotary shaker at 4°C for 72 h.

The GdnHCl extracts were placed in Spectra Por 3 dialysis tubing and dialyzed extensively against water at 4°C. Following dialysis, the extracts were lyophilized, suspended in 500 μl of 20 m*M* Tris (pH 8.0), applied to a 20 cm × 2.5 cm I.D. column of Sephadex G-25-150 and eluted with 20 m*M* Tris (pH 8.0). All the eluate which contained material that absorbed at 280 nm was pooled and lyophilized.

The EDTA extracts were lyophilized without dialysis and desalted using PD-10 columns following the manufacturer's instructions with 20 m*M* Tris (pH 8.0) as the eluent.

SDS-PAGE

SDS-PAGE was conducted using gels that were 0.75 mm thick with 12% polyacrylamide (separating) and 4% polyacrylamide (stacking). A discontinuous Tris–glycine buffer system [6] was used with a Bio-Rad Labs. Mini-Protean II electrophoresis cell.

Desalted, lyophilized extracts were suspended in 500–1000 μl of 20 m*M* Tris (pH 8.0) and analyzed for protein content by the Bio-Rad Labs. protein assay method. SDS-PAGE dissociation buffer was mixed with the sample (1:1, v/v) and the mixture was boiled for 5 min. Approximately 1.5 μg of protein were placed in each well of the gel. One well of each gel contained 5 μl of low-molecular-weight standard (Bio-Rad Labs.) that had been diluted 1:100 (v/v) with sample buffer.

Electrophoresis was run at constant voltage of 200 for 45–60 min. Bands were then detected with silver stain following manufacturer's instructions (Bio-Rad Labs.).

The migration distances of each standard protein and the migration distance of the tracking dye was measured and R_F values were calculated. R_F values were also calculated for the unknown proteins and the relative molecular weights (M_r) of the unknowns were calculated from the R_F valves of the standards.

HPLC

HPLC analyses of extracts were performed using a Waters Model 440, HPLC absorbance detector Model 712 WISP autoinjector, Model 501 pump and Model 740 data module. The detector was set to monitor absorbance at 280 nm, the range was set to 0.02, the integrator attenuation was set to 32 and the chart speed was set to 10.

HPLC analyses were conducted by a modification of the method of Takagi [7]. The modifications made were to use two TSK G3000 SW columns (30 cm × 7.5 mm I.D.) and 0.1 *M* phosphate elution buffer (pH 6.0) containing 0.1 *M* sodium nitrate and 0.1% SDS at a flow-rate of 0.3 ml/min.

The same sample suspension as described for SDS-PAGE was mixed with an equal volume of 10% SDS–mercaptoethanol (3:1, v/v) and boiled for 5 min. A volume of the suspension calculated to contain 5–10 μg of protein was injected into the HPLC system. The M_r values of the NCPs were calculated from a plot of retention time *vs.* log M_r of low-molecular-weight standards which has been treated in the same manner. Fig. 1 shows an elution pattern for the low-molecular-weight standards used and a plot of log M_r of the standards *vs.* retention time.

Partially, purified NCP samples (a gift from Dr. C. W. Prince) were prepared as described above and injected into the HPLC system.

RESULTS

Fig. 2 shows a typical SDS-PAGE pattern for GdnHCl extracts from newborn pups and their dams. In the GdnHCl extract from the pups, there were discrete bands with M_r *ca.* 100 000, 60 000, 50 000, and 15 000. In the GdnHCl extract from the dams, the major discrete band was at M_r 15 000. In GdnHCl extracts from both the dams and the pups, there were many minor bands with M_r between 100 000 and 15 000.

Fig. 3 is a typical SDS-PAGE pattern for EDTA

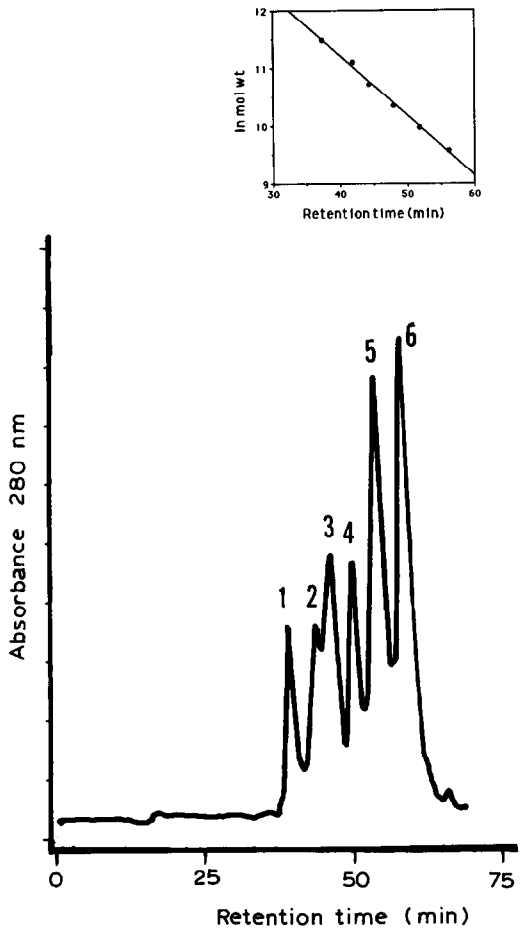

Fig. 1. SDS-HPLC of low-molecular-weight standards obtained with two TSK-G3000 columns in tandem. Standards used were the low-molecular-weight standards obtained from Bio-Rad Labs. Molecular weights of (1) 97 400, (2) 66 200, (3) 45 000, (4) 31 000, (5) 21 500 and (6) 14 400. Inset: plot of log M_r of the standards *vs.* retention time; $y = 15.358 - 0.1036x$.

extracts from newborn rat pups and their dams. In the EDTA extract from the pups, the major discrete bands had M_r 60 000, 20 000 and 10 000. The relative lightness of the bands in the EDTA extract reflects the lack of protein in the EDTA extract from newborn rat pups. In the EDTA extract from the dams, the major discrete bands had M_r 60 000, 45 000 and 15 000. As with the GdnHCl extracts, there were many minor bands with M_r between 100 000 and 15 000.

With 12% gels, no protein remained in the stack-

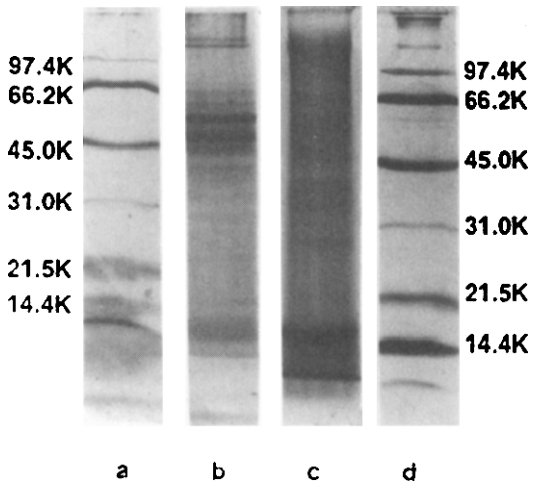

Fig. 2. SDS-PAGE profiles of GdnHCl extracts from tibias and femurs of newborn rat pups and their dams: 12% polyacrylamide gels were run with discontinuous Tris–glycine buffer systems and bands were detected with silver staining. Lanes: a = low-molecular-weight standards (Bio-Rad Labs.); b = GdnHCl extract from newborn rat pups; c = GdnHCl extract from dams; d = low-molecular-weight standards (Bio-Rad Labs.). K = kilodalton.

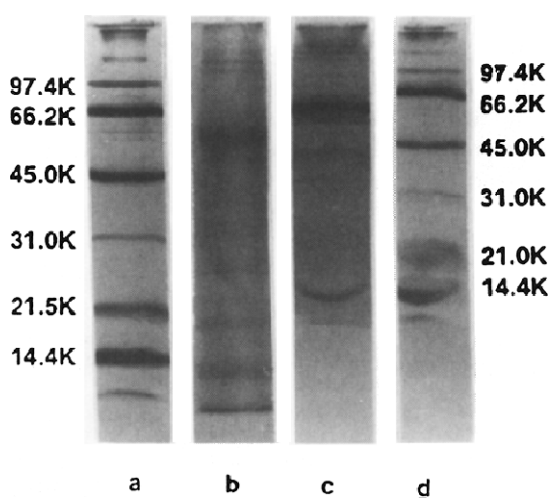

Fig. 3. SDS-PAGE profiles of EDTA extracts from tibias and femurs of newborn rat pups and their dams: 12% polyacrylamide gels were run with discontinuous Tris–glycine buffer systems and bands were detected with silver staining. Lanes: a = low-molecular-weight standards (Bio-Rad Labs.); b = EDTA extract from newborn rat pups; c = EDTA extract from dams; d = low-molecular-weight standards (Bio-Rad Labs.).

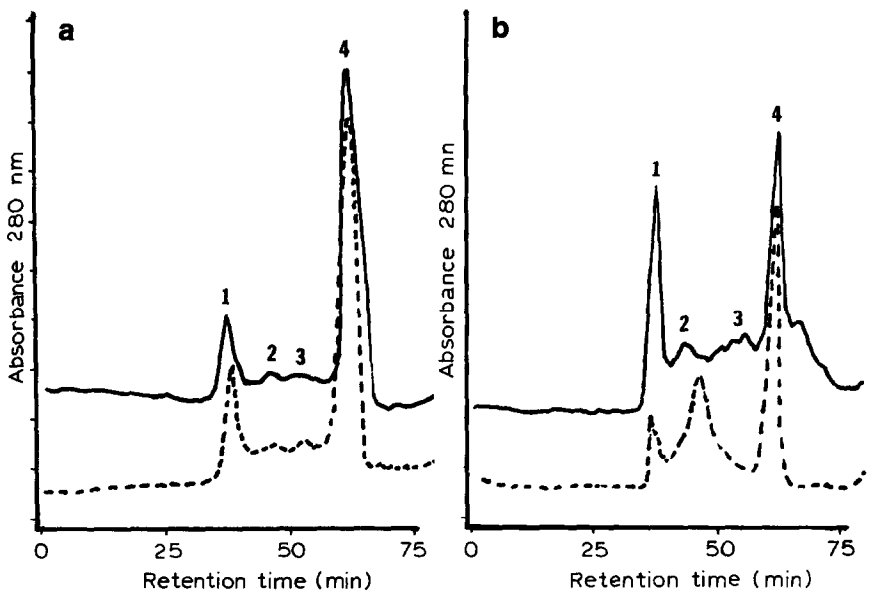

Fig. 4. SDS-PAGE profiles of (a) GdnHCl and (b) EDTA extracts from femurs and tibias of newborn rat pups (dashed lines) and their dams (solid lines). HPLC was performed using two 30-cm TSK 3000 columns in tandem, absorbance was monitored at 280 nm and the flow-rate was 0.3 ml/min. Between 5 and 10 μg of protein were injected. Peaks (M_r): 1 = 115 000; 2 = 55 000; 3 = 30 000; 4 = 12 000.

ing gels and the minimal staining at the dye front suggested that little proteolytic degradation had occurred.

Fig. 4 shows examples of SDS-HPLC profiles of GdnHCl and EDTA extracts from newborn pups and their dams. The percentage of the total peak area and M_r represented by each of the major peaks are given in Table I. From both extracts, there were major peaks with retention times of 35 and 57 min (peaks 1 and 4, respectively). There was also a set of poorly resolved peaks with retention times between 43 min (peak 2) and 50 min (peak 3). For GdnHCl extracts from the bones of mature rats, peak 1 represented 13.4% of the total peak area and peak 4 was 69.5% of the total peak area (Table I). For EDTA extracts from the bones of mature rats, peak 1 represented 13.2% of the peak area and peak 4 49.5% of the total peak area (Table I). For GdnHCl extracts from the bones of the pups, peak 1 was 18.1% and peak 4 was 57.4% of the total peak area.

TABLE I

PERCENTAGE OF TOTAL PEAK AREA AND APPARENT MOLECULAR WEIGHT (M_r) REPRESENTED BY EACH PEAK FROM SDS-HPLC OF GdnHCl AND EDTA EXTRACTS OF BONES OF NEWBORN RAT PUPS AND THEIR DAMS

n = 4 or 5.

Peak	M_r	GdnHCl extract		EDTA extract	
		Pups	Dams	Pups	Dams
1	115 000	18.1 ± 5.8	13.4 ± 3.2	27.5 ± 5.2	13.2 ± 3.3
2	55 000	–	6.4 ± 3.2	26.4 ± 5.2	32.6 ± 3.3
3	30 000	22.1 ± 5.8	8.2 ± 3.2	–	–
4	12 000	57.4 ± 5.8	69.5 ± 3.2	43.5 ± 5.2	49.5 ± 3.3
5	5000	0.2 ± 5.8	1.0 ± 3.6	–	0.5 ± 3.3

From the EDTA extract from the bones of the pups, peak 1 was 27.5% and peak 4 was 43.5% of the total peak area (Table I). Peak 2 was absent in the GdnHCl extract from the bones of the pups and was 26.4% of the total peak area in the EDTA extract from the bones of the pups. Peak 3 was 22.1% of the total peak area in the GdnHCl extract from the bones of the pups and was absent in the EDTA extract.

In some of the samples from both the GdnHCl and the EDTA extracts, a small peak with a retention time of 67 min (peak 5) was evident. This peak had M_r between 2000 and 4000 and might represent proteolytic degradation products. That this peak averaged less than 1% of the total peak area suggested that little degradation of the samples had occurred.

The SDS-HPLC method was also used on partially purified NCP samples. The partially purified sample that contained largely osteonectin had the major peak at 45 min which corresponded to peak 2. The partially purified sample that contained largely proteoglycans had its major peak at the same retention time as peak 1 (35 min).

DISCUSSION

The advantages of the method described in this paper include (1) a relatively short extraction time of about 1 week, which may explain the lack of obvious degradation, (2) the use of generally accepted GdnHCl and EDTA extraction procedures to separate NCPs associated with the non-mineralized phase and the mineralized phase of bone and (3) the use of 5–10 μg of protein for SDS-HPLC and 1.5 μg of protein for SDS-PAGE. These amounts of protein can be obtained from five neonatal rats and three mature rats.

There were clear differences in NCP profiles between GdnHCl and EDTA extracts in mature rats; there differences were not as apparent in neonatal rats, which may reflect the relative lack of mineralization in pups [8]. In the GdnHCl extract from the dams, the major bands detected by SDS-HPLC had M_r *ca.* 100 000 and 15 000, whereas with the pups there were discrete bands with M_r *ca.* 100 000, 60 000, 50 000 and 15 000 (Fig. 2). The SDS-PAGE of the EDTA extracts (Fig. 3) was simpler than that of the GdnHCl extract (Fig. 2). The greater simplicity of the gels of EDTA extracts is in agreement with data of Termine *et al.* [1]. The major components of the EDTA extract of fetal calf bones have been reported to have M_r values of 61 000–63 000, 31 000 and 22 000–26 000 [1], whereas those of rat compact bone have been reported to be above 50 000 [3]. The major bands detected by SDS-PAGE of the EDTA extracts from the newborn rat pups in this study had M_r *ca.* 60 000, 20 000, and 10 000.

The profiles of the NCPs obtained using SDS-HPLC corresponded closely with those from SDS-PAGE. In all samples there was a peak with M_r *ca.* 115 000 in both PAGE and HPLC; this protein, termed peak 1, can be tentatively identified as proteoglycans. In most of the samples, the proteins in the M_r range 30 000–60 000 were poorly resolved in both PAGE and HPLC. Nevertheless, it is possible to note differences in the relative amounts of peak 2 (M_r = 55 000), which can be tentatively identified as largely osteonectin, and peak 3 (M_r = 34 000) between the GdnHCl and EDTA extracts (Fig. 4). These differences were especially pronounced in the extracts from the dams. Peak 4 (M_r = 12 000) is a major band in both HPLC and PAGE and presumably represents the γ-carboxy glutamic acid proteins. Although the M_r of peak 2 is closer to that of α-2HS glycoprotein than that of osteonectin, the retention time of peak 2 was the same as that of a partially purified NCP preparation that contained largely osteonectin. That the gla proteins represented a larger percentage of the total NCPs in the bones of the dams than in the bones of the pups was expected [9] and is in agreement with the results of Kuboki *et al.* [4].

When these samples were applied to a single TSK-G3000 HPLC column in the absence of SDS, one or two peaks were obtained (data not shown). The addition of SDS partially resolved those peaks and lengthening the column further increased the resolution. It is possible that resolution could be further increased by further lengthening the column and/or monitoring absorbance at 230 or 210 nm in addition to 280 nm.

The use of methods such as that described in this paper to obtain NCP profiles does not allow the identification or quantification of specific NCPs, but does allow qualitative comparison of treatments and can indicate possible NCPs for further study.

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