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Semipreparative isolation of collagen types I, II, III and V by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and electroelution

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Abstract

A simple method for the isolation of α-chains of different collagen types was developed. The procedure involves sodium dodecyl sulfate–polyacrylamide gel electrophoresis followed by electroelution of separated and defixed collagen α-chains. Collagen types I, II, III and V from different porcine tissues were recovered in high quantity (>95%) and purity (>98%) as evidenced by amino acid analysis. The procedure can be used for sample quantities smaller than required for conventional methods, e.g. chromatographic procedures.

Keywords: Electroelution; Collagens; Proteins

1. Introduction

Collagens are the predominant structural proteins of all connective tissues. The preparative isolation of pure collagen types generally involves several steps of differential extraction and salt precipitation followed by chromatographic procedures [1–3]. At present, sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) is the most widely used analytical procedure for the identification of collagen chains. In contrast to most other proteins, collagens do not migrate on SDS–PAGE exactly according to their molecular mass. Due to the high content of imino acid residues (about 20%), collagen peptides might have a more rigid conformation in SDS than common proteins migrating on SDS–PAGE according to their molecular masses [4]. The isolation of different collagen α-chains for further analysis usually requires chromatographic methods [4,5].

PAGE in the presence of SDS is a common and useful method for the separation of proteins and large peptides. It also appears attractive to use electrophoretically separated peptides for further analysis, and the combination of electrophoresis with electroelution is widely used for the pure separation and characterization of various non-collagenous proteins [6,7]. However, to our knowledge, this approach has not been used for collagenous proteins. Furthermore, since dyes can interact in different

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ways with different proteins, the protein stain (Coomassie Blue) may interfere with both the recovery rate following electroelution of different collagens as well as with the subsequent analysis of the proteins or peptides [7]. Thus, electroelution of proteins from SDS–PAGE comprises two crucial steps; the total elution of proteins and a high recovery rate. The recovered proteins may be submitted to further analytical methods, such as peptide mapping, amino acid analysis and amino acid sequencing. Specific chemical cleavage, e.g. of collagen α-chains at methionine residues with cyanogen bromide (CNBr) can also be performed after separation by SDS–PAGE and electroelution of the respective peptides. In addition, the cleavage products, after digestion with collagenase, can be isolated and characterised by combination of SDS–PAGE and electroelution.

In this paper, we present a simple procedure for the isolation and purification of α-chains of different fibrillar collagens (types I, II, III and V). This method represents an alternative to the chromatographic separation of collagens under denaturing conditions. We also describe modifications of the electroelution procedure, resulting in higher transfer rates from SDS–polyacrylamide gels. The electroelution of collagen α-chains from SDS gels to a dialysis tube was quantitated and conditions were optimized for different collagen types with different molecular masses.

The presented method may greatly facilitate the analysis of collagen and its breakdown products from skin, cartilage, tendon and bone obtained from patients with various connective tissue diseases, e.g. inherited or acquired skin disorders, metabolic bone diseases and fibrotic processes of various organs.

2. Materials and methods

2.1. Bone, cartilage, skin and tendon samples

Samples were obtained from the articular bone, rib, articular cartilage, skin and kneecap-tendon of seven pigs. All samples were cleaned from adhering tissue and washed several times with water, methanol, chloroform–methanol (2:1, v/v) and freeze-dried.

2.2. Extraction of collagens

The bone, cartilage, skin and tendon samples were powdered under liquid nitrogen using a stainless steel homogenizer and were suspended in 0.5 *M* EDTA (ethylenedinitrilotetraacetic acid; Merck, Darmstadt, Germany). The bone powder (about 2–3.5 g dry mass) was demineralized by dialysis at 4°C against repeated changes of 0.5 *M* EDTA (pH 7.4) for ten days and against 0.5 *M* acetic acid containing 0.2 *M* NaCl (pH 2) for an additional three days. After homogenization, the tissue powder (about 0.5–1 g dry mass) was suspended in 0.5 *M* acetic acid containing 0.2 *M* NaCl (pH 2). Limited pepsin digestion was used to solubilize collagen. Demineralized bone, cartilage, skin and tendon samples were stirred in a pepsin solution (0.1 mg/ml, pH 1.8 in 0.5 *M* acetic acid, 0.2 *M* NaCl; Boehringer Mannheim, Germany) at 4°C for 24 h. After centrifugation at 90 000 *g* (1 h, 4°C), supernatants were neutralized and stored at −20°C. This digestion procedure was repeated five times and all neutralized supernatants were pooled [1–3]. Collagens I, II, III and V of pepsin extracts were precipitated by 4.5 *M* NaCl. After salt precipitation and centrifugation (at 90 000 *g*, 1 h, 4°C), the pellets were resuspended in 0.5% acetic acid and exensively dialyzed against 0.5% acetic acid and then were lyophilized.

2.3. SDS–PAGE

Following pepsin cleavage, 200–500 μg of lyophilized collagen were dissolved to a final concentration of 2 mg/ml in sample buffer (63 m*M* Tris, 2% SDS, 10% glycerol, 0.01% bromophenol blue, pH 6.8) [9]. After heating at 95°C for 3 min and quenching on ice, separation of collagen types and their α-chains was perfomed on SDS–polyacrylamide [7] slab gels, using 4% (w/v) polyacrylamide for the running gel and 6% (w/v) for the stacking gel (standard sized slab gels; 16.5 cm×17.7 cm×3 mm). Separation of collagen types and α-chains was carried out in the presence or absence of 2-mercaptoethanol in the sample buffer as well as

with delayed reduction. The bands of individual collagen α-chains were cut out of the gel.

2.4. Electroblotting

Follwing electrophoretic separation, protein bands were blotted onto a poly(vinylidene difluoride) (PVDF) membrane (Immobilon-P transfer membrane, 0.45 μm pore size). The gels were washed in a transfer buffer (40 m*M* 6-amino-N-hexanacid, 5% methanol, 0.1% SDS, pH 7.2) for 5 min, to reduce the amount of bicine [N,N-bis(2-hydroxyethyl)glycine] and Tris. During this time, the PVDF membrane was rinsed with 100% methanol for 2 min and stored in deionized water. The gels were sandwiched between a sheet of PVDF membrane and several sheets of blotting paper (Schleicher and Schuell, 580 nx, 600 mm, Dassel, Germany) and were asseambled in a blotting apparatus (S and S CarboGlas, Schleicher and Schuell). Protein bands were blotted onto the PVDF membrane for 2.5 h at 100 mA and 4°C. After electroblotting, the Immobilon membrane was washed in deionized water for 5 min, stained with 0.1% Coomassie blue in 50% methanol for 5 min and then was destained in 50% methanol, 10% acetic acid for 5–10 min and air-dried. The blotted protein bands were cut out with a clean scalpel and pooled in an Eppendorf minifuge tube (1.5 ml). The pooled bands were soaked in 1 ml of elution buffer (50 m*M* Tris, 2% SDS, 1% Triton X-100, pH 9.0) and were shaken overnight at room temperature. After elution of α-chains, the membrane slurry was transferred into an ultra spin Microfilter (molecular mass cut-off of 30 000; Roth, Karlsruhe, Germany) and then placed in the microcentrifuge (Hermle Z 231 M, Gosheim, Germany). The salts in the elution buffer were removed by centrifugation at 4000 *g* for 30 min at room temperature.

2.5. Electroelution

After separation of the different collagen α-chains by electrophoresis, individual bands were visualized by staining with Coomassie blue (0.125% Coomassie Blue R-250, 4% methanol and 20% trichloroacetic acid added with 1 l of deionized water) for 10 min, then destained with 7% acetic acid. Bands were cut out with a scalpel and placed into an Eppendorf tube. The gels were soaked in defixing buffer [6 m*M* urea, 192 m*M* bicine, 25 m*M* Tris, 0.2% SDS and 5 m*M* DTT (dithiothreitol)] for 0.5 h at room temperature to solubilize the separated collagen chains. After incubation, the gel slices were equilibrated in electrophoresis buffer (192 m*M* bicine, 25 m*M* Tris and 1.73 m*M* SDS, pH 8.5) for 5 min, cut into small pieces and placed into an electroelution glass tube (Model 422 Electro-Eluter, Bio-Rad, Munich, Germany). Elution was performed at 10 mA/glass tube constant current for 2 h at room temperature. The different α-chains were transferred from the gel slice through the frit into the membrane cap and retained by a dialysis membrane molded into the cap (molecular mass cut off of 15 000).

3. Desalting by ultrafiltration and SDS and Coomassie Blue removal by acetone precipitation

After electroelution, samples were lyophilized and subsequently dissolved in 0.5 ml of 1% acetic acid and 0.5% SDS. Each membrane, loaded with an individual, concentrated α-chain sample, was transferred to an ultra-spin Microfilter (Roth, 30 000 molecular mass cut-off) and placed into a microcentrifuge. The salts (Tris, bicine and parts of the Coomassie Blue) contained in the elution buffer were removed by centrifugation at 4000 *g* for 30 min at room temperature. Following centrifugation, the ultrafilter was removed from the centrifuge and the concentrate (100–200 μl) was recovered from the membrane. Four volumes of cold acetone (−20°C) were added to the gel eluent and the samples were allowed to precipitate for 30 min in a freezer (−20°C). The tubes were then centrifuged for 10 min at 15 000 *g*, the acetone supernatants were aspirated and the tubes were inverted to drain. Samples were washed with 1 ml of 80% acetone and 0.5% acetic acid buffer (−20°C) to remove traces of residual SDS and Coomassie Blue.

3.1. Amino acid analysis

Amino acid analysis was performed on a Beckman 6300 automated analyzer using post-column derivatization with ninhydrin. The eluent was monitored

at 570 and 440 nm, respectively. The relative content of hydroxylysine (Hyl) and hydroxyproline (Hyp) per mole of fractionated collagen was expressed as the ratio of Hyl/(Hyl+Lys) and Hyp/(Hyp+Pro), respectively.

4. Results and discussion

Using a combination of electrophoretic separation by SDS–PAGE, defixation and electroelution, more than 95% of pepsin-solubilized collagen α-chains were recovered. This recovery rate from the polyacrylamide gel was determined by comparing the amount of collagen loaded on the gel with the amount of collagen obtained after electroelution and this was markedly higher than that achieved by blotting of electrophoretically separated collagens onto a PVDF membrane without using a defixing buffer (about 10% recovery rate) and in the same range as that seen with blotting plus defixation (own data, not shown). However, the method described omits the blotting step and does not involve any detergent (e.g. Triton X-100), which, in contrast, is necessary for the blotting procedure and has to be removed from the collagen prior to further analysis of it. For these reasons, the combination of electrophoresis and electroelution is much more practicable and less time-consuming than the blotting procedure. The standard protocol for the preparative separation and isolation [1–6] of individual collagen α-chains is rather complicated: it involves fractional salt precipitation of pepsin-solubilized material to achieve separation of collagen types, followed by chromatographic isolation of α-chains [8]. In contrast, the method described here requires only one single precipitation with 4.5 *M* NaCl, immediately followed by electrophoretic separation of α1(I), α2(I), α1(II), α1(III) and α1(V) and electroelution. The amount of the material needed is as little as 0.5 to 1 g (wet mass) of tissue or 10 to 100 mg of protein, respectively, depending on the source. Conventional, more complicated protocols require much larger sample quantities [1–3]. Thus, biopsy material, e.g. of skin and bone, may now be used for detailed biochemical analyses of the collagenous matrix.

Since glycine cannot easily be removed from the buffer and interferes with the amino acid analysis of α-chains from collagens, we modified the electrophoresis and electroelution conditions and chose instead the larger homologue of glycine, bicine [10,11].

The flow diagram shown in Fig. 2 summarizes the different procedures involved in the semi-preparative isolation of collagen types I, II, III and V by SDS–PAGE and electroelution. A SDS–polyacrylamide gel electrophoretogram of the α-chains of types I, II, III and V collagen is shown in Fig. 1. The α-chains of type I collagen demonstrate unusual behaviour in that α2(I) chains have greater electrophoretic mobility than α1(I) chains of the same molecular mass (95 000). Separation of collagen types I, III and V and their α-chains was carried out both under reducing and non-reducing conditions. For the identification of collagen III, delayed reduction was performed (Sykes). Therefore, the α-chains of collagen III do not migrate as far as the α1(I) chains (apparent molecular masses of 140 000–160 000). The α-chains of type V collagen have a molecular mass of approximately 125 000. We have found that, after defixation of collagen α-chain gel bands, 2 h is the optimal electroelution time (>98% efficiency).

For recovery of α-chains from SDS-containing solutions, several procedures have been described

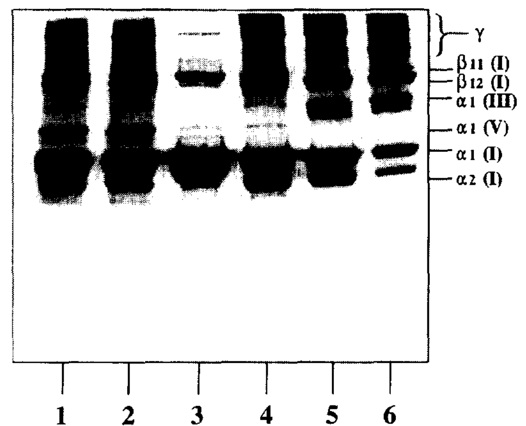

Fig. 1. SDS–PAGE of different collagen types showing their α-, β- and γ-components. The samples were prepared in a concentration of 2 mg/ml of sample buffer (1 *M* Tris, 2% SDS, 10% glycerol, 0.25% bromophenol blue, pH 6.8). After heating at 95°C for 3 min, the samples were quenched on ice. Lanes: 1=standard collagen of fetal bone; 2–5=α-, β_{12}–β_{11}- and γ-components from pig collagen extracts. 2=articular bone; 3=rip cartilage; 4=rip bone; 5=skin and 6=standard collagen of fetal skin.

including ion-exchange chromatography, protein precipitation, high-performance liquid chromatography, fast protein liquid chromatography and gel filtration (Sephadex G-25 NAP-columns, Pharmacia, Freiburg, Germany; own unpublished data). Fig. 2 summarizes the sequence of the experimental procedures used. The bicine, Tris and traces of elution buffer (SDS and Coomassie Blue) were removed by centrifugation at 5000 g for 30 min at room temperature. Following centrifugation, the Ultrafilter was removed from the centrifuge and the concentrate was recovered from the membrane. Acetone precipitation was used to concentrate α-chains of collagen from SDS-containing buffers. Almost all (99.5%) of the SDS and Coomassie Blue can be found in the acetone phase, with less than 0.5% of the SDS and Coomassie Blue remaining in the protein pellet [12]. The presence of residual SDS (0.5%) is essential for

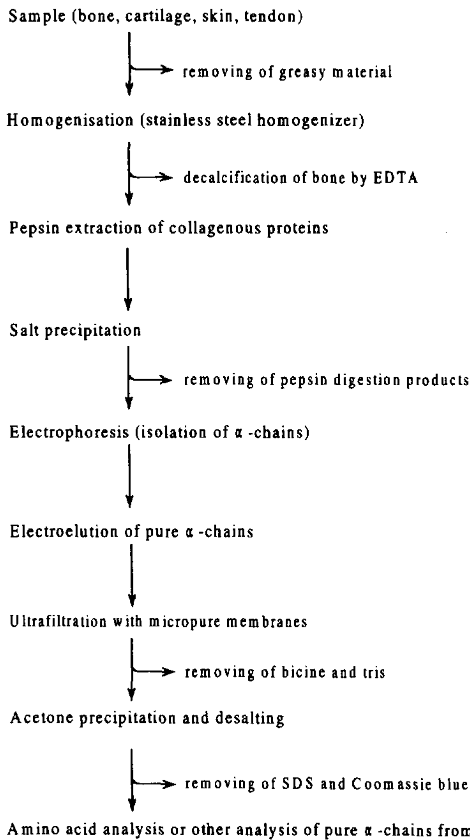

Fig. 2. Flow diagram for the isolation and purification of α-chains of different fibrillar collagens (I, II, III and V).

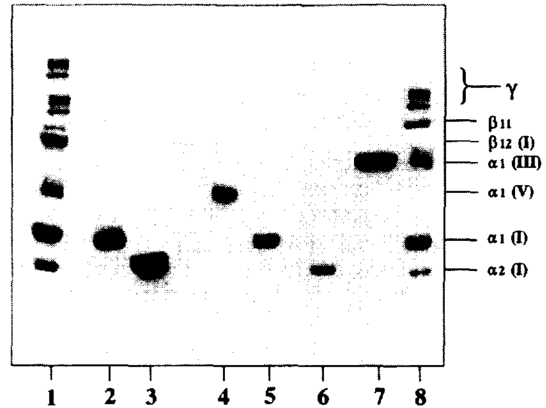

Fig. 3. Re-electrophoresis showing individual α1(I)-, α2(I)-, α1(III)- and α1(V)-chains after electroelution of electrophoretically separated collagen. 1=standard with collagen types I and V from calf bone; 2=α1(I) from bone; 3=α2(I) from bone; 4=α1(V) from bone; 5=α1(I) from skin; 6=α2(I) from skin; 7=α1(III) from skin and 8=standard with collagen types I and III from fetal skin.

the removal of the proteins from the filter membrane, since SDS inhibits the binding of α-chains to the latter (own unpublished data). The re-electrophoresis of the obtained material is shown in Fig. 3. The purity of the recovered material was >98%. Table 1

Table 1
Amino acid composition[a] of pig collagen α-chains isolated by SDS–PAGE after pepsin digestion

Amino acid	[α1(I)]	[α2(I)]	[α1(II)]	[α1(III)]	[α(V)]
Hyp	99	91	98	120	103
Asp	46	45	44	40	48
Thr	17	21	21	19	21
Ser	34	30	25	36	34
Glu	72	72	88	82	91
Pro	130	118	116	110	104
Gly	337	340	339	343	335
Ala	114	112	105	95	47
Val	20	30	15	22	26
Met	6	7	8	2	11
Ile	7	9	9	9	18
Leu	21	31	27	23	43
Tyr	2	2	1	1	2
Phe	13	11	12	12	11
His	4	7	4	2	11
Hyl	8	13	18	7	34
Lys	26	20	20	28	18
Arg	44	41	50	51	43

[a] Values are expressed as residues/1000 total amino acid residues.

Table 2
Hydroxylysine (Hyl) and Hydroxyproline (Hyp) residues in different collagen α-chains isolated by SDS–PAGE after pepsin digestion

Chain	Hyl/Hyl+Lys	Hyp/Hyp+Pro
[α1(I)]	0.235±0.021	0.432±0.017
[α2(I)]	0.394±0.023	0.435±0.064
[α1(II)]	0.474±0.024	0.458±0.019
[α1(III)]	0.20±0.012	0.522±0.033
[α1(V)]	0.654±0.008	0.498±0.021

summarizes the results of the amino acid analysis from various types of α-chains from collagen I, II, III and V from pig skin, cartilage and bone. The relative content of Hyl and Hyp per mole of fractionated α-chains of types I, II, III and V collagen expressed as the ratio of Hyl/(Hyl+Lys) and Hyp/(Hyp+Pro), respectively, is shown in Table 2. The isolated collagen α-chains can readily be used for further analysis, e.g. digestion with bacterial collagenase, CNBr cleavage, amino acid analysis, determination of the reducible difunctional cross-linked compounds (dihydroxylysinonorleucine, DHLNL and dehydrolysinonorleucine, HLNL). The amino acid analysis may be of diagnostic significance in connective tissue related disorders, e.g. Ehlers-Danlos Syndrome type VI [13–15], osteoporosis [16,17] and osteogenesis imperfecta [18,19].

In conclusion, we present an efficient procedure for the separation and isolation of α-chains of different collagen types from various connective tissues. The procedure requires only small tissue quantities, is easy to perform and is characterized by a high recovery rate and high purity of the separated material. The obtained collagen α-chains can be used for further analysis.

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