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Journal of Chromatography B, 790 (2003) 277–283
JOURNAL OF CHROMATOGRAPHY B
www.elsevier.com/locate/chromb

Simple and rapid chromatographic purification of type V collagen from a pepsin digest of porcine intestinal connective tissue, an unmanageable starting material for conventional column chromatography

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Abstract

A chromatographic method is described for purification of type V collagen, a minor constituent in extracellular matrix, from a pepsin digest of porcine intestinal connective tissue. The starting material was a viscous and turbid solution even after centrifugation. Direct application of the sample to a commercially available DEAE-cellulose column resulted in clogging. On the other hand, type V collagen, $[\alpha1(V)]_2\alpha2(V)$ form, was successfully captured by a filter paper-based DEAE-cellulose column chromatography and purified by a subsequent commercially available cation-exchange medium without clogging. This is a vast improvement over previously described salt fractionation methods.

Keywords: Collagens; Proteins; Cellulose

1. Introduction

Liquid chromatography is a most effective tool for the separation of proteins of interest from a biological source. Before liquid chromatographic separation, a preliminary fractionation, occasionally multi-step, is necessary to remove undesirable contaminants such as particulate matter, lipids or other substances which may result in fouling or clogging of subsequent chromatographic system. Recovery of the protein often decreases in the preliminary fractionation step. In addition, time-consuming preliminary fractionation may be a stumbling block for scale-up of the protein purification. Recently, we developed a filter paper-based (FPB) DEAE-cellulose for simultaneous clarification and fractionation of the particle-containing feedstocks, which may be a good substitute for the conventional preliminary fractionation step [1].

The objective of the present study is to confirm that the FPB DEAE-cellulose column chromatography can simplify the protein purification from the start material which requires tedious preliminary fractionation before the chromatographic procedure. As a model, FPB DEAE-cellulose column chroma-

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1570-0232/02/$ – see front matter
doi:10.1016/S1570-0232(03)00083-7

tography is applied to the purification of type V collagen. The type V collagen is a member of fibril-forming collagens (Types I, II, III, V, and XI) and consists of three subunit chains designated α1(V), α2(V), and α3(V). At least two submolecular species, $[\alpha1(V)]_2\alpha2(V)$ and α1(V)α2(V)α3(V), have been described [2,3]. It has been demonstrated that the type V collagen has unique biological activities such as inhibition of cell-substratum adhesion of vein endothelial cell and some cancer cell lines [4–13] and can also bind some biologically active materials such as heparin, insulin, osteonectin, thrombospondin, macrophage colony-stimulating factor and so on [14–20]. The type V collagen has been prepared by the tedious multi-step salt fractionation and subsequent chromatographic procedure [21–23]. The present study demonstrates that the type V collagen can be directly captured by the FPB DEAE-cellulose column chromatography without the tedious salt fractionation and can be purified by the subsequent chromatographic procedure using a commercially available cation exchanger without clogging.

2. Experimental

2.1. Regents

Urea (biochemical grade) and acetonitrile (HPLC grade) were obtained from Nacalai Tesque (Kyoto, Japan). Porcine pepsin was obtained from Sigma (St Louis, MO, USA). Other chemicals were of analytical grade or better.

2.2. Chromatographic media and columns

Bakerbond WP-CSX 40 μm was obtained from J.T. Baker (Phillipsburg, NJ, USA). An Express-Ion Exchanger D was obtained from Whatman (Kent, UK). A disposable polypropylene column (12.5 mm I.D.) with 10-ml sample reservoir and an Econo column (100×25 mm I.D.) were purchased from Bio-Rad Labs (Hercules, CA, USA). HR 5/10 and MM-1550 glass columns were obtained from Pharmacia Biotech (Uppsala, Sweden) and Yamazen (Osaka, Japan), respectively. Bakerbond WP-Butyl and -PEI HPLC columns (50×4.6 mm I.D.) were from J.T. Baker. A Vydac C_{18} 218 TP54 reversed-phase column was purchased from Grace Vydac (Hesperia, CA, USA). FPB DEAE-cellulose was prepared by the method described previously [1].

2.3. Chromatographic apparatus

The HPLC system consisted of a Shimadzu (Kyoto, Japan) LC-9A pump, an FCV-9AL gradient valve unit, a DGU-2A degasser unit, an SPD-6AV variable-wavelength detector and a Rheodyne Model 7125 sample injector with a 5-ml sample loop. The preparative LC system consisted of a Yamazen Model 600A pump, an ATTO (Tokyo, Japan) Gradicon III valve controller, a Gradi AC-5905 valve unit and an Eyela (Tokyo, Japan) UV-9000 variable-wavelength detector.

2.4. Preparation of crude collagen sample

Pepsin-solubilized collagen was prepared from porcine intestine by the method described previously [1]. Porcine intestine was obtained from Nippon Meat Packers Co. (Tokyo, Japan). The inner content was washed away with tap water. The cleaned intestine was cut into small pieces (approximately 10 mm×10 mm) with scissors and minced. The minced intestine was pre-treated with cold 0.1 *M* NaOH to solubilize non-collagenous proteins and exclude the effects of endogenous enzymes on collagens. The residue was digested with porcine pepsin in 10 m*M* HCl at 5 °C overnight in an enzyme substrate ratio of 1:100 and then centrifuged at 10 000 *g* for 20 min. The pepsin digestion cleaves only non-triplehelical domains of collagen and consequently solubilizes the collagen molecules from tissue [2,3]. The solubilized collagen has native triplehelical structure. This digestion was repeated three times. The supernatants were combined and mixed with 0.3 vol. of 10 *M* urea solution containing 250 m*M* NaCl and then added to 1 *M* Tris base to give pH 8.2. Finally, distilled water was added to give 2 *M* urea and 50 m*M* NaCl and used as the sample for the FPB DEAE-cellulose chromatography.

2.5. Capturing step of type V collagen by the FPB DEAE-cellulose column chromatography

The FPB DEAE-cellulose was suspended in 50 m*M* Tris–HCl buffer, pH 8.2 containing 2 *M* urea and 50 m*M* NaCl and stirred vigorously in a Waring blender. Small particles generated by the vigorous stirring were removed by squeezing in cotton cloth and then re-suspended by gentle shaking in the Tris buffer and packed into the column (12.5 or 25 mm I.D.) to 10 cm in height. The crude collagen sample was applied to the sample reservoir of the column. The columns were washed with the Tris buffer and the adsorbed proteins were subsequently eluted with 0.3 and 1.0 *M* NaCl in the buffer. For comparison, the commercially available fast flow type of DEAE-cellulose (Express-Ion Exchanger D) was packed into the same column according to the manufacturer's instructions. The same collagen sample was applied to the Express-Ion column.

2.6. Purification of type V collagen by Bakerbond WP-CSX column chromatography

Two sizes of the Bakerbond WP-CSX columns (100×5 and 150×50 mm I.D.) were equilibrated with 40 m*M* Tris–HCl buffer, pH 8.2 containing 50 m*M* NaCl and 2 *M* urea. The small column was connected to the HPLC system and the large one to the preparative LC system. For the small column, adsorbed proteins were eluted with the buffer containing 170 and 500 m*M* NaCl at 1 ml/min. For the large column, the adsorbed proteins were eluted with a linear gradient of NaCl from 50 m*M* to 1.0 *M* (1.5 l) at 50 ml/min.

2.7. Isolation of type V collagen subunits

The purified type V collagen was dissolved in 20 m*M* Tris–HCl buffer, pH 7.0 containing 2 *M* urea and heated at 50 °C for 5 min to resolve into subunits. The subunits were isolated by anion-exchange HPLC using the Bakerbond WP-PEI equilibrated with the 20 m*M* Tris–HCl buffer, pH 7.0 containing 2*M* urea as described previously [23]. The elution was carried out by a linear gradient of NaCl from 0 to 350 m*M* in the buffer over 20 min at 1 ml/min. Elution was monitored at 230 nm.

To remove non-volatile buffer and salt from the protein for amino acid analysis, reversed-phase HPLC was carried out. The sample was applied with a 5-ml sample loop to the Bakerbond WP-Butyl column equilibrated with 10% acetonitrile containing 0.1% trifluoroacetic acid (TFA). After elution of the buffer components, protein was eluted by increasing the acetonitrile from 10 to 80% over 10 min at 1 ml/min.

To isolate the α1 subunit of type XI collagen, if present, which can assemble in a molecule with α1 and α2 chains of type V collagen, reversed-phase HPLC using the Vydac C_{18} 218 TP 54 column was carried out by the method of Niyibizi and Eyre [24]. The elution was carried out with a binary gradient of 0.1% TFA (A) and a mixture of acetonitrile–*n*-propanol (3:1) (B). Proteins were eluted by increasing the solution B from 16 to 30% over 50 min at 1 ml/min.

2.8. Other analytical procedures

Amino acid analysis was carried out by the method of Bidlingmeyer et al. [25] with a slight modification [23].

Sodium dodecylsulfate–polyacrylamide gel electrophoresis (SDS–PAGE) was carried out by the method of Laemmli [26] using 7.5% gel. Protein bands in the gel were developed with Coomassie Brilliant Blue R250. The staining intensity of protein bands was evaluated using a Master Scan 486 (Scanalytics, Billerica, MA, USA). Type V collagen content was estimated on the basis of staining intensity of the α1(V) chain.

3. Results

3.1. Isolation of type V collagen

The pepsin-digest of the porcine intestine was a milk-white turbid solution with high viscosity even after centrifugation at 10 000 *g* for 20 min. The collagen sample could not be clarified by filtration using a cellulose acetate membrane (3.0 μm) due to rapid clogging. Direct application of the collagen sample to the column (100×12.5 mm I.D.) packed with the commercially available anion exchanger

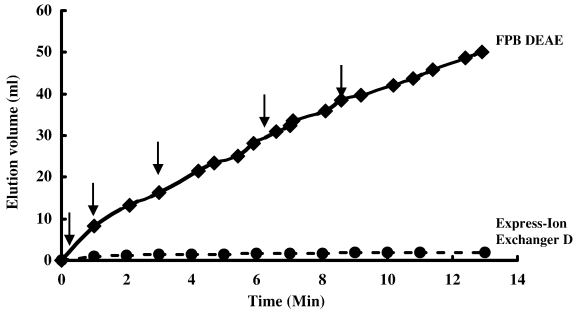

Fig. 1. Flow-rate of the crude collagen sample from the columns (100×12.5 mm I.D.) packed with FPB DEAE-cellulose and Express-Ion Exchanger D. Ten milliliters of the crude collagen sample were applied to both columns and then each 10 ml sample was given at the time arrows indicated.

(Express-Ion Exchanger D) also resulted in rapid clogging (Fig. 1). On the other hand, more than 50 ml of the crude sample could be applied to the FPB DEAE-cellulose column (100×12.5 mm I.D.) with sufficient flow-rate. As shown in Fig. 2, type I collagen was recovered in the non-adsorbed fraction (peak A) and type V collagen and pepsin were eluted with 0.3 *M* (peak B) and 1.0 *M* NaCl (peak C), respectively. The α1(V), α2(V) subunits and their oligomers are detected by SDS–PAGE analysis. On the other hand, no significant amount of the α3(V) subunit was detected.

The effluent from the FPB DEAE-cellulose column was transparent, indicating that effective clarification occurred. The type V collagen-rich fraction obtained by the FPB DEAE-cellulose column was dialyzed against the equilibrium buffer of the Bakerbond WP-CSX column and applied to the small Bakerbond WP-CSX column. As shown in Fig. 3, types I and V collagen were eluted with 170 m*M* (peak B) and 500 m*M* NaCl (peak C), respectively. Coupling with the FPB-DEAE cellulose chromatography, highly purified type V collagen can be isolated in a few hours.

On the basis of these results, scale-up of the type V collagen purification was carried out. Two hundred milliliters of the crude collagen sample (0.12–0.25 mg/ml) was applied to the large FPB DEAE-cellulose columns (100×25 mm, I.D.) and type V collagen was eluted with 100 ml of the buffer containing 300 m*M* NaCl. Twenty pieces of the column were simultaneously used. The pooled type V collagen-rich

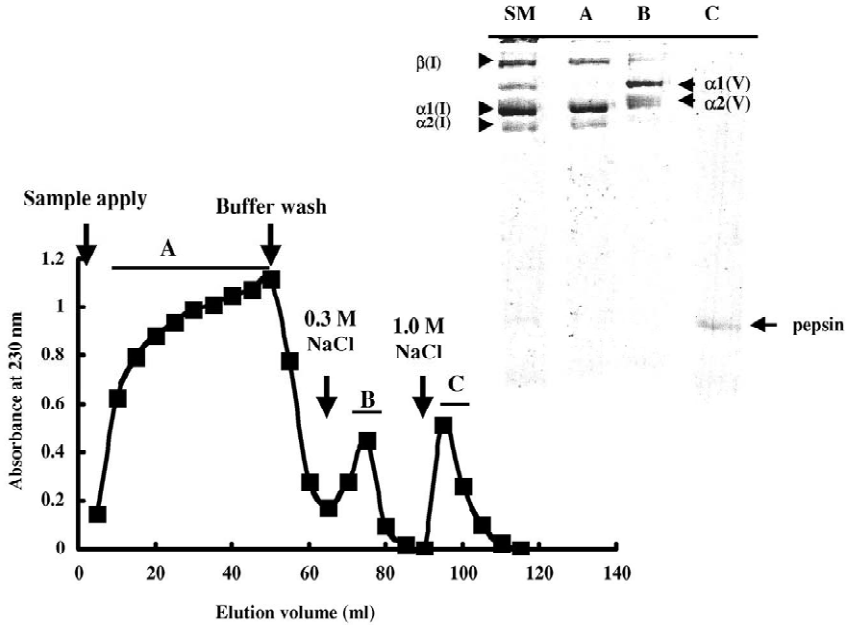

Fig. 2. Capturing of type V collagen from the crude collagen sample by the FPB DEAE-cellulose column (100×12.5 mm I.D.) chromatography. SM, start material. β represents covalently cross-linked dimers of the subunits. High molecular materials over β chains are oligomers of the subunit chains.

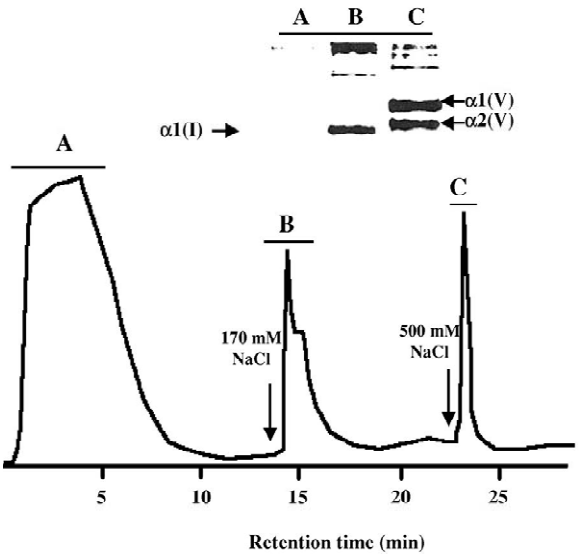

Fig. 3. Purification of type V collagen by the small Bakerbond WP-CSX column (100×5 mm I.D.). High-molecular mass materials in the type V collagen fraction (C) are cross-linked oligomers of type V collagen subunits. Arrows indicate start of the elution with 170 m*M* and 500 m*M* NaCl.

fraction (2 l) was diluted with 2 vol. of the equilibrium buffer and applied to the large Bakerbond WP-CSX column (150×50 mm, I.D.) by the pump. As shown in Fig. 4, type I collagen was eluted in the non-adsorbed fraction (fraction A) and type V collagen was eluted by the gradient of NaCl (fractions B and C). The type V collagen in the effluent could be recovered as a precipitate by dialysis against water or mixing with cold ethanol to give 35% (v/v). In the present study, up to 0.4 g of type V collagen was isolated by the single run of the Bakerbond WP CSX chromatography by application of 2 l of the type V collagen-rich fraction obtained by the FPB DEAE-cellulose chromatography. The recovery of type V collagen by the Bakerbond WP-CSX column chromatography was between 70 and 90% on the basis of staining intensity of α1(V) chain resolved on the SDS–PAGE.

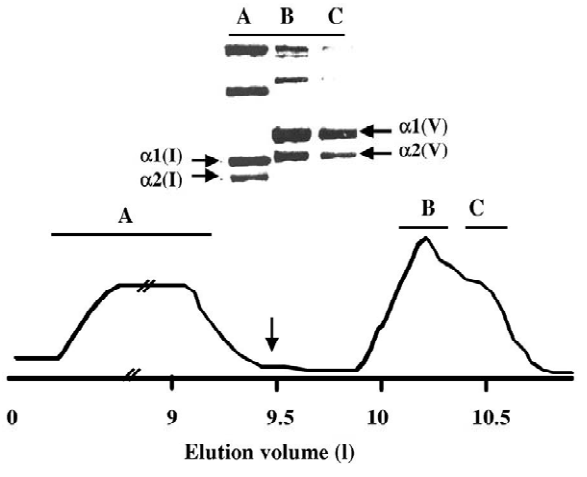

Fig. 4. Purification of type V collagen by the large Bakerbond WP-CSX column (150×50 mm I.D.). Arrow indicates start of the gradient program.

3.2. Characterization of the purified type V collagen

As shown in Fig. 5, two subunit chains with electrophoretic mobility corresponding to the α1(V) and α2(V) chains were resolved by the anion-exchange HPLC. As shown in Table 1, they show typical composition of the type V collagen α1(V) and α2(V) chains, respectively [2].

Nyibizi and Eyre [24] demonstrated the occurrence of cross-type heterotrimer between type V and XI collagen, designated as α1(V)α1(XI)α2(V). It is difficult to distinguish the α1(XI) chain from the

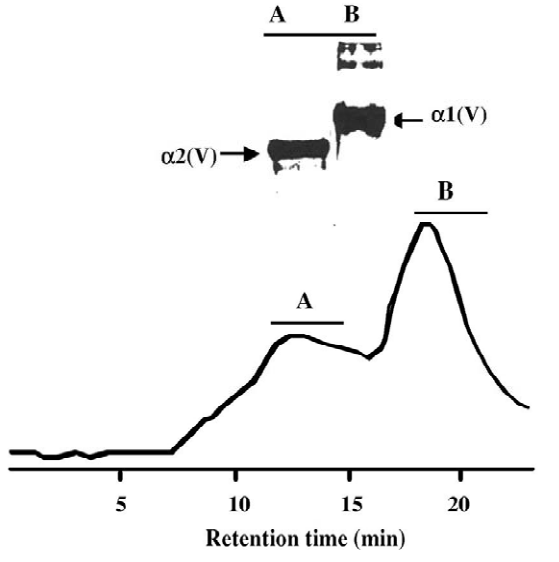

Fig. 5. Isolation of subunits of type V collagen by the Bakerbond WP-PEI HPLC column.

Table 1
Amino acid composition of α1(V) and α2(V) chains (residue/1000 residues)

Amino acid	α1(V)		α2(V)	
	Porcine[a]	Human [26]	Porcine[a]	Human [26]
Asp	40	49	42	50
Glu	131	100	109	89
Hyp	103	115	107	109
Ser	27	23	36	34
Gly	353	332	354	331
His	5	6	9	10
Arg	40	40	52	48
Thr	21	21	27	29
Ala	31	39	39	54
Pro	136	130	121	107
Tyr	2	4	2	2
Val	15	17	23	27
Met	8	9	13	11
Ile	15	17	9	15
Leu	27	36	25	37
Hyl	29	36	17	23
Phe	10	12	9	11
Lys	8	14	8	13

[a] Means for three determinations.

α1(V) by SDS–PAGE and ion-exchange chromatography. Therefore, the subunits were also fractionated by the method of Niyibizi and Eyre [24]. As shown in Fig. 6, a small peak (peak B) was detected after the elution of α1(V) and α2(V) (peak A), which corresponds to the elution position of α1(XI). However, SDS–PAGE analysis revealed that the peak B contained no collagenous protein. Together with these data, the present type V collagen preparation predominantly consisted of a molecule designated as $[\alpha1(V)]_2\alpha2(V)$, a most prevalent molecular form of type V collagen [2,3].

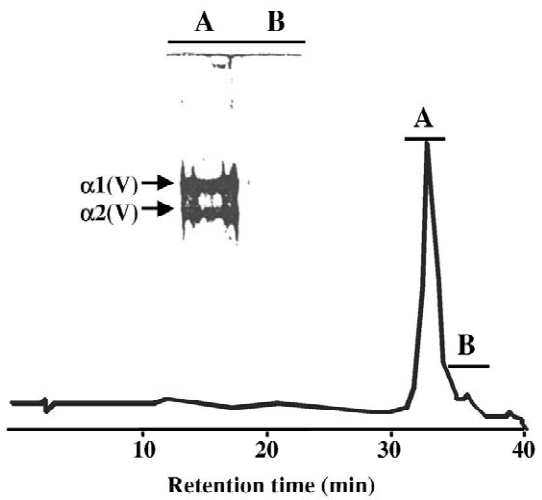

Fig. 6. Fractionation of the subunits of type V collagen by reversed-phase HPLC according to the method of Niyibizi and Eyre [23].

4. Discussion

Type V collagen has been isolated by multi-step salt fractionation, which is tedious and laborious work and unsuitable for scale-up [21–23]. Therefore, only a small amount (normally few milligrams level) of type V collagen can be obtained after laborious work (few days). For further elucidation of the biological activity of type V collagen, development of a rapid and simple isolation procedure for type V collagen is necessary. In the present study, we used the FPB DEAE-cellulose column chromatography instead of the salt fractionation method. By using the FPB DEAE-cellulose column (12.5 or 25 mm I.D.), a few milligrams of type V collagen can be captured from the starting material in a short time (a few hours) without clogging (Figs. 1 and 2). No special solvent delivery system is required for the FPB DEAE-cellulose column chromatography. Therefore, even 20 pieces of the column can be simultaneously used without difficulty to obtain a much greater amount of type V collagen. In addition, it is worth noting that effective clarification occurred by passing through the FPB-DEAE cellulose column. Thereby, the effluent containing type V collagen can be applied to subsequent cation-exchange chromatography using the commercially available high-performance chromatographic media for further purification.

The sample for the FPB DEAE-cellulose column chromatography can be obtained just by mixing the pepsin-digest of connective tissue dissolved in 10 m*M* HCl with the urea, Tris-base and NaCl solutions. Furthermore, we found that type V collagen can be adsorbed to the Bakerbond WP-CSX in the presence of less than 170 m*M* NaCl under the same pH conditions for the FPB DEAE-cellulose chromatography. The type V collagen-rich fraction eluted with 300 m*M* NaCl from the FPB DEAE-cellulose column can be used for the subsequent

cation-exchange purification step just by diluting with 2 vol. of the equilibrium buffer for the second chromatography. The simple pretreatment without dialysis step would also facilitate large-scale isolation of type V collagen based on the present approach. It has been demonstrated that type V collagen has submolecular forms. On the basis of electrophoretic, HPLC and compositional analyses, the type V collagen isolated from the porcine intestine by the present procedure can be identified as the most prevalent $[\alpha1(V)]_2\alpha2(V)$ form. Co-assembly of type XI collagen subunit was not detected in the present preparation. By introducing the FPB DEAE-cellulose column chromatography instead of salt fractionation, a vast improvement over the previous purification method can be achieved.

The FPB DEAE-cellulose can be re-used after washing with 0.1 *M* NaOH. In the present application, the used FPB DEAE-cellulose was suspended in 0.1 *M* NaOH. The milk-white supernatant was removed by decantation until it became colorless. Then the resin was extensively washed with water and equilibrated with the starting buffer. A similar result was obtained using the regenerated media. However, the FPB DEAE-cellulose can be prepared inexpensively using commonly available glassware and reagents (sodium hydroxide, hydrogen chloride, sodium chloride and 2-chlorotriethylamine) [1]. Then we usually discard it after a single use.

The FPB DEAE-cellulose used in the present study was prepared manually. Partial characteristics of the media have been published [1]. Additional optimization of fiber length, ion-exchange capacity of the FPB DEAE-cellulose and column size should be carried out for the further improvement of performance. If equivalent media are prepared on an industrial scale, it would be a powerful tool for a large-scale capturing procedure of proteins from biological sources.

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