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Review

Preparative procedures and purity assessment of collagen proteins

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Abstract

Collagens represent a large family (25 members identified so far) of closely related proteins. While the preparative procedures for the members that are ubiquitous and present in tissues in large quantities (typically fibre and network forming collagens types I, II, III, IV and V) are well established, the procedures for more recently discovered minor collagen types, namely those possessing large non-collagenous domain(s) in their molecule, are mostly micropreparative and for some collagenous proteins even do not exist. The reason is that the proof of their existence is based on immunochemical staining of tissue slices and nucleic database searching. Methods of preparation and identification of constituting α-polypeptide chains as well as collagenous and non-collagenous domains are also reviewed. Methods for revealing non-enzymatic posttranslational modifications (particularly of the fibre forming collagen types) are briefly described as well.

Keywords: Preparative chromatography; Reviews; Collagens; Proteins

Contents

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1. Introduction

Since the last extensive review about collagenous proteins [1] the number of the members of the collagen superfamily has grown from 19 to 25 [2–8]. Most of these proteins are ubiquitous in vertebrate tissues and the proportion of different types varies within broad limits. The whole set of collagen proteins can be divided into two subfamilies: those which form large structures like tendons, skin, cartilages, etc., are typical in having most of the molecule built up from the typical collagenous sequence (Gly-Pro-X tripeptides); those which are present at considerably lower concentrations (and frequently codistributed with the former ones) possess large non-collagenous terminal domains and/or interrupted helices. It is beyond the scope of this review to present detailed description of the structure of individual members of the collagen family; for more information the potential readers of this review are directed to the classical paper of Prockop and Kivirikko [1], in which the structural arrangements of collagens type I–XIX are surveyed. About the additional ones (beyond type XIX) only scattered information in the literature is available.

2. Classical (abundant) collagen types (I–V)

Before applying any preparative chromatographic procedure collagens must be isolated from the source tissue. The most abundant ones, typically collagen types I–V are poorly soluble (if at all) and, consequently, they have to be brought to the solution either by limited enzyme digestion (types I, II, IV) [9–13], by disulphide bond cleavage (type III) [14] or by some other, less frequently used operation [solubilization by means of sodium dodecylsulphate (SDS)] [15]. Limited proteolytic cleavage is based on the fact that the insolubility of the protein causing cross-links are located in the terminal, non-collagenous domains which are susceptible to cleavage by a number of rather non-specific proteases, typically pepsin; while the main collagenous core of the molecule (possessing typical collagenous sequences) remains intact and can be cleaved by specific collagenases only [16]. Disulphide bonds cleavage can be applied to those collagens in which the polymerization is partly due to the disulfide bonds formed by cysteine residues (typically collagen type III; most of the other collagens are devoid of this amino acid within the collagenous core; however, the non-collagenous domains—extensions—usually possess this amino acid).

As demonstrated in Table 1 the physico-chemical properties of different collagen types offer the possibility to replace the complex chromatographic and precipitation methods just described by salting out procedures [17].

Collagen obtained by limited pepsin digestion and purified by DEAE-cellulose chromatography is used as starting material. It has to be stressed that removing of proteoglycans is essential for fraction precipitation (see above). Collagen type III is salted out by increasing the NaCl concentration to 1.7 *M* (some times also 1.5 *M* is used). Type I is precipi-

Table 1
Survey of molecular properties and precipitation conditions of different collagen types (according to Deyl and Adam [17])

Type	Molecular formula	M_r of α-chain	NaCl (*M*)	
			Precipitation at acidic pH	Precipitation at neutral pH
I	$[\alpha_1(I)]_2\alpha_2(I)$	95	0.7–0.9	2.6
I trimer	$[\alpha_1(I)]_3$		0.7–0.9	4.0
II	$[\alpha_1(II)]_3$	95	0.7–0.9	3.5–4.0
III	$[\alpha_1(III)]_3$	100–95	0.7–0.9	1.5–1.7
IV	$[\alpha_1(IV)]_3$ $[\alpha_1(IV)]_2\alpha_2(IV)$	180–75	1.2	1.7–2.0
V	$[\alpha_1(V)]_2\alpha_2(V)$	200–130	1.2	3.6–4.5
VI	$\alpha_1(VI)\alpha_2(VI)\alpha_3(VI)$	240–140	2.0	
VII	$[\alpha_1(VII)]_3$	>170		
VIII	$[\alpha_1(VIII)]_3$	61		
IX	$\alpha_1(IX)\alpha_2(IX)\alpha_3(IX)$	85	2.0	
X	Unknown	59	2.0	
XI	$[\alpha_1(XI)]_2\alpha_2(XI)$	95	1.2	

From Ref. [30] with permission.

tated with 2.4 *M* $CaCl_2$. The collagen precipitates have to be washed at least once with the buffer in which they were formed, dissolved in Tris, 0.3 *M* NaCl, pH 7.4 (adjusted with HCl as necessary) and dialysed against the same buffer. Further increase in salt concentration up to 3.5–4.0 *M* in the supernatant from which collagen type I was precipitated results in the precipitation of collagen type V.

Fraction precipitation is a simple approach which, regretfully, allows only partial separation of the coprecipitated collagen species. Consequently the precipitate is always contaminated and chromatographic techniques have to be exploited in a subsequent separation step (for review see Ref. [18]).

Relatively the most easily accessible collagen type is type I and its soluble form, called acid-soluble collagen (incompletely crosslinked portion of collagen type I). A very small proportion of collagen type I can be extracted from collagen-rich tissues (typically tendons or skin) also by neutral salt solutions extraction (neutral salt collagen). Both acid and neutral salt soluble collagens represent non-polymerized type I collagen. All preparations obtained by neutral salt extraction and always heavily contaminated by codistributed non-collagenous proteins. The insoluble residue obtained by the procedure in Fig. 1 represents a mixture of cross-linked collagen types I and III.

Further separation of collagen types I, III and V (which are co-distributed in tissues) can be done according to the flow-sheet shown in Fig. 2.

Collagen type II is in most cases prepared from cartilage. In accordance with what has been said above, limited proteolytic digestion has to be applied. In general, other proteases besides pepsin can be used for this purpose [19]. In our hands pronase proved most versatile. A flow sheet of this procedure is presented in Fig. 3.

2.1. Chromatography on soft sorbents and gels

Chromatographic procedures are used either for direct isolation of collagens from tissue extracts or for further purification of the precipitated materials. Typically, collagen type II can be obtained without proteolysis directly from embryonic cartilage [20]. The neutral salt extract (1 *M* NaCl) is passed through a 15×2.5-cm DEAE-cellulose column equilibrated with 0.2 *M* NaCl, 0.05 *M* Tris buffer, pH 7.5, by which procedure the co-extracted proteoglycans are removed. The column temperature is kept at 5 °C and the column is eluted at a flow-rate of 90 ml/h. The simplified flow sheet of this procedure is presented in Fig. 4.

Preliminary purification of collagenous materials by DEAE-cellulose chromatography is not limited to preparation of collagen II only; this step appears necessary for further collagen fractionation as long

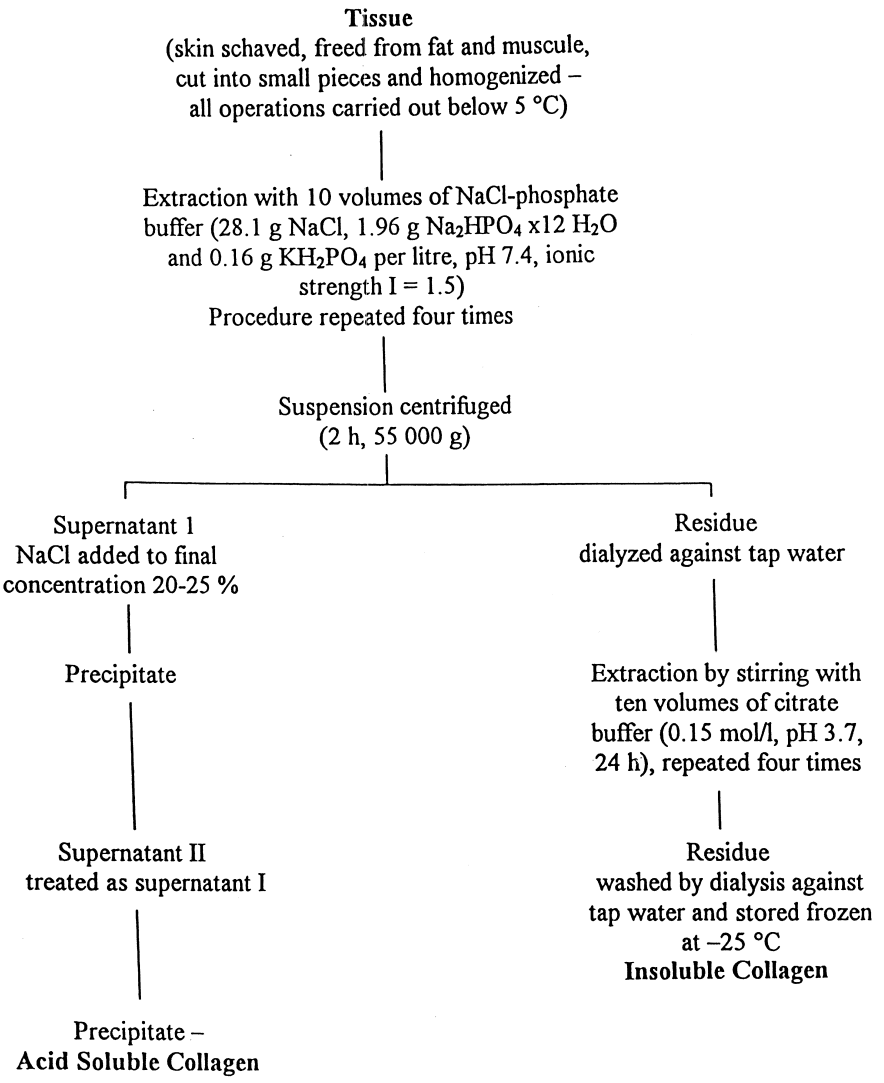

Fig. 1. General strategy for isolating collagenous material from soft tissues [18].

as proteoglycans, owing to their interaction with collagen, have disturbing effects upon the separation of individual collagen species. The generally applicable procedure runs as follows: lyophilized collagen is dissolved in 0.2 *M* NaCl, 0.05 *M* Tris–HCl, pH 7.5, and applied to a DEAE-cellulose column (Whatman DE-52) which is equilibrated with the same buffer and cooled to preserve the collagen triple helix. On elution the effluent is monitored at 230 nm and as soon as no absorbing material is eluted any more, the eluting solvent is changed abruptly to 1.0 *M* NaCl, 0.05 *M* Tris–HCl buffer, pH 7.5, and elution with the latter buffer is continued until another peak is eluted from the column. The first peak representing purified collagen is freed from non-volatile solutes by dialysis against 1% acetic acid and lyophilized. The other peak represents proteoglycans (and possibly collagens possessing large glycosidic domains) (Fig. 5) [20].

For the separation of collagen type V from a mixture of collagen I and III DEAE-cellulose chromatography is applicable as well [21]. The procedure

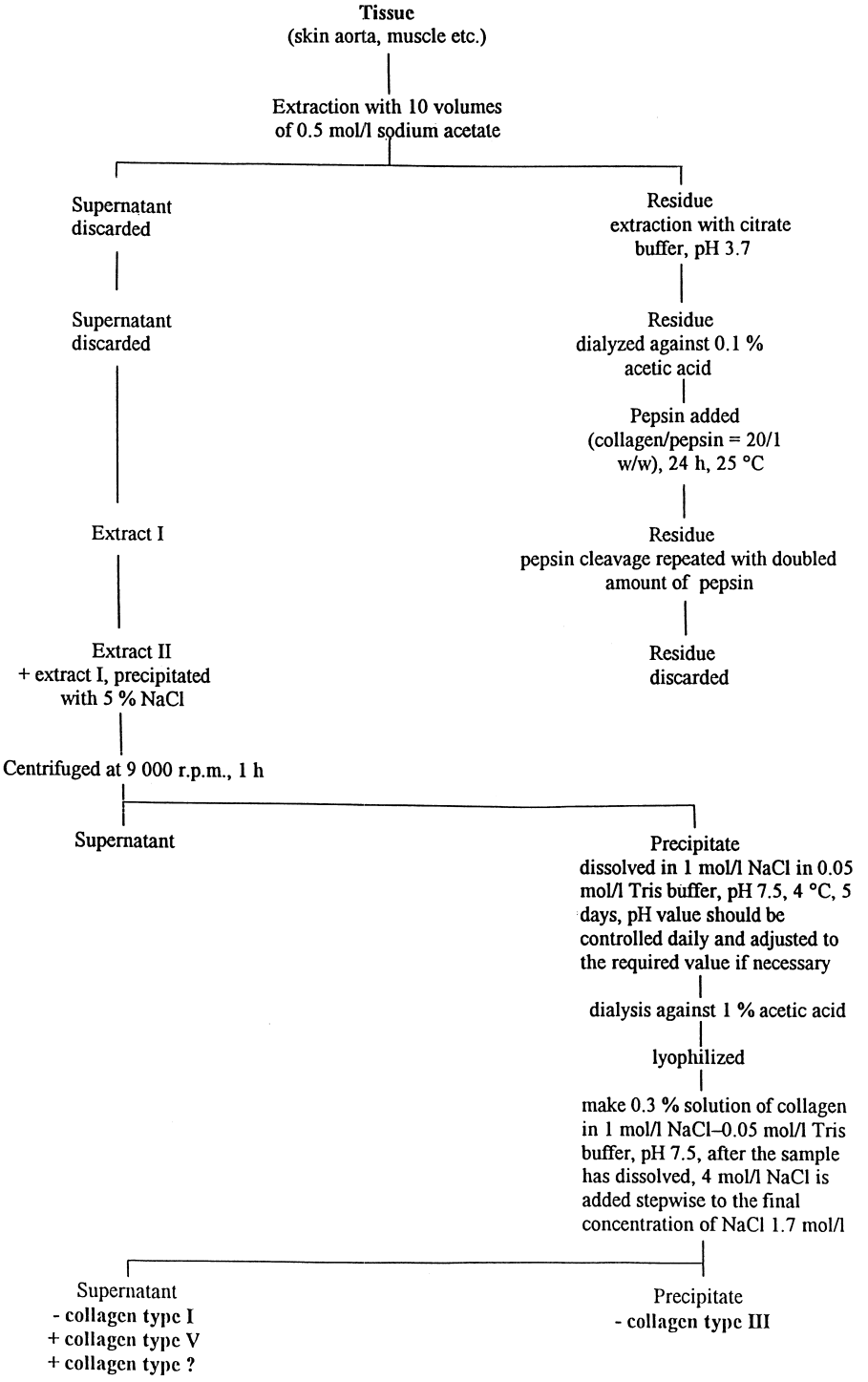

Fig. 2. Flow sheet for collagen types I (+V) and III preparation [18].

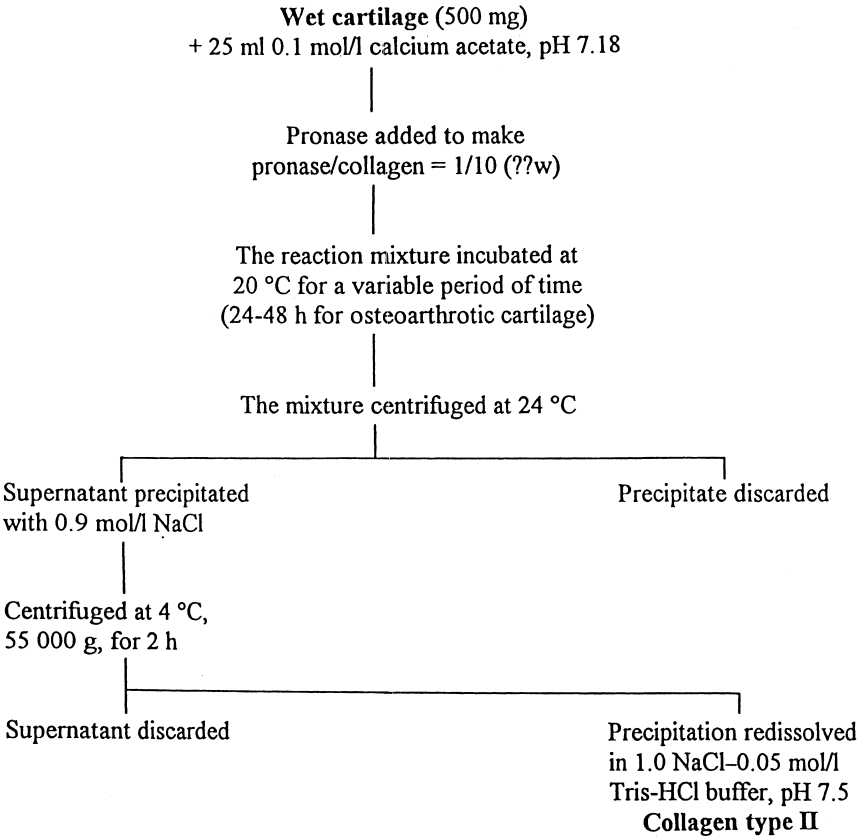

Fig. 3. Flow sheet for collagen type II preparation from cartilage (pronase treatment of the parent tissue) [18].

is a slight variation of that just described. The crude collagen sample is dissolved in 0.02 *M* NaCl, 0.05 *M* Tris–HCl buffer, pH 7.5, 2 *M* with respect to urea and applied to a 20×2.5-cm DEAE-cellulose column, refrigerated and equilibrated with the same buffer. At the time when no further UV-absorbing material is detectable in the eluate (230 nm), the eluting solvent is changed to a linear gradient from 0.02 *M* NaCl in 0.05 *M* Tris–HCl buffer, pH 7.5, to 0.3 *M* NaCl in 0.05 *M* Tris–HCl, pH 7.5 (both buffers made 2 *M* with respect to urea). The first peak observed is formed by a mixture of collagen types I and III, the peak eluted after the gradient has been introduced is mainly collagen type V.

Alternatively, separation of collagen type III from collagen type I can be done by molecular sieving [22]. In this case the separation of collagen type III is based on the presence of the disulphide bonds in this collagen type. A Bio-Gel A-1.5m column (150× 4.5 cm) is used with 1 *M* $CaCl_2$ in 0.05 *M* Tris–HCl buffer, pH 7.5, as eluent. The UV absorbance profile exhibits three maxima corresponding to M_r 300 000, 200 000 and 100 000. On the contrary to the previously described separation, this one is carried out with denatured collagen. Samples are heated to 43 °C to ensure denaturation before the material is applied to the column. Owing to the presence of disulphide bonds, collagen type III is present in the fastest peak (as the parent α-chains are disulfide bonded yielding the $[\alpha_1(III)]_3$ polymer of M_r 300 000), where also chain polymers (γ) of collagen type I are present. Peaks emerging with a higher retention volume contain depolymerized collagen type I (chain monomers and dimers).

The final proof of the presence of collagen type III in the fast running fraction can be done by reduction and alkylation. For this purpose 20 mg of the isolate are dissolved in 10 ml of 8 *M* urea, 0.1 *M* with

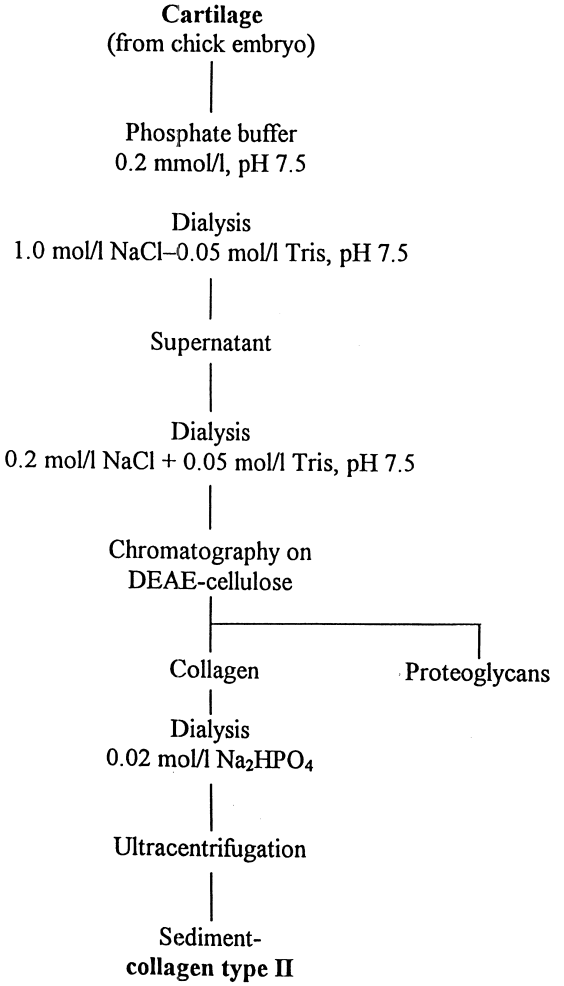

Fig. 4. Flow sheet for collagen type II preparation from embryonic cartilage [18].

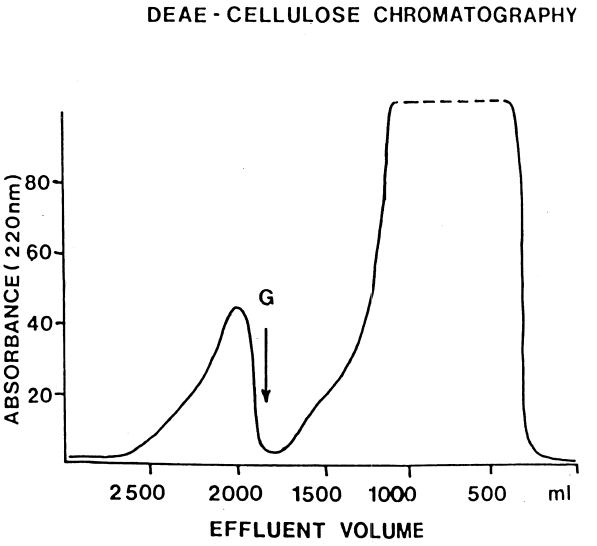

Fig. 5. DEAE-cellulose chromatography of the peptic digest of renal collagen used for the removal of proteoglycans. The arrow indicates the change in the eluting buffer from 0.2 *M* NaCl to 1 *M* NaCl (0.05 *M* Tris, pH 7.5). The peak with low retention volume represents collagen, the other represents proteoglycans. From Ref. [18] with permission.

respect to Tris, pH 8.0, attained by addition of Tris free base. The reaction mixture is flushed with nitrogen and 2-mercaptoethanol is added (1.42 ml/l). The mixture is incubated at 37 °C for 4 h and then the solution is made 0.02 *M* with respect to iodoacetate. The reaction mixture is left for another 45 min at room temperature in darkness [11]. After desalting by dialysis and subsequent lyophilization the whole sample is dissolved in the eluting buffer and subjected to a second Bio-Gel A-1.5m chromatography. A 150×3.0-cm column is used with the same operating conditions as those specified above. The resulting single peak (under optimal circumstances) represents reduced and alkylated collagen type III α chain.

The already-mentioned fraction precipitation can be also exploited for chromatographic separation—zone precipitation chromatography (Fig. 6) [23,24]. A step-wise sodium chloride gradient is used to redissolve and precipitate collagens from the inter-bead space of a molecular sieve. Fundamental to zone precipitation is the fact that protein molecules are too big to enter the inter-bead space. They in fact move faster than the mobile phase which penetrates the bead and has a longer distance to traverse. When the protein in the solution outruns the precipitant concentration where it is soluble, it occurs finally at a higher salt concentration at which it precipitates from the solution. The solvent concentration, however, after some time drops down. As a matter of fact it becomes so low that the precipitated protein goes back into solution. Since the precipitate is very fine, dissolution proceeds at a high rate. Finally the protein is eluted at the concentration which is characteristic for its dissolution. It is worth stressing that according to SDS–polyacrylamide gel electrophoresis (PAGE) type I collagen is represented here

by three species which very likely reflects the fact that pepsin treatment produces heterogeneous population of collagen type I molecules.

In practice the column is partly equilibrated in 30% and partly in 25% NaCl. The sample is applied and the column eluted with equal volumes of 20, 15, and 10% saline. The eluate is collected in a fraction collector and the final wash of the column is done with 1% saline. The scheme of the separation is seen in Fig. 6. By this method it is possible to separate type I, type II, type III and type V collagens side by side. The NaCl gradient can be replaced by ammonium sulphate with good results [23].

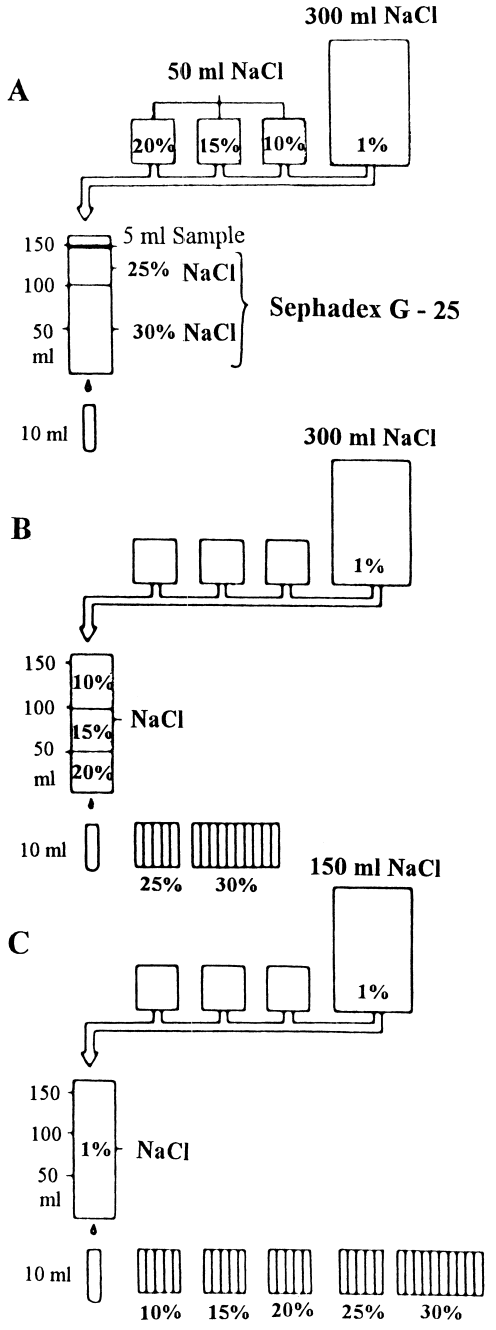

Fig. 6. A schematic view of zone precipitation chromatography. All the salt solutions and sample are in 50 m*M* Tris–HCl, pH 7.5, buffer. (A) A sample of 5 ml in buffered 1% NaCl is layered on top of the column. The column is equilibrated in buffered 30% NaCl (100 ml) followed by 25% NaCl (50 ml). Separate 50-ml volumes of 20% NaCl, 15% NaCl, 10% NaCl and 300 ml of 1% NaCl are prepared and stand ready for application to the column. (B) Fifty ml of the column elution are collected in 12-ml fractions. First, 50 ml of the 20% NaCl solution were applied followed by 50 ml of 15% NaCl solution and then 50 ml of 10% NaCl solution. The 300 ml of 1% NaCl solution stands ready for application. (C) Three hundred ml of the column elution are collected in 12-ml fractions; 150 ml of the 1% NaCl solution pass through the column and 150 ml are left to be applied to the column. The optical density of each fraction is read at 230 nm along with its conductivity. From Ref. [23] with permission.

2.2. Chromatography on rigid sorbents

The classical preparative procedures make use of soft gels or soft sorbents not withstanding high pressures. Of the rigid sorbents used for the preparation of the common collagen types (I, II and III) cyanopropyl phases have been introduced as early as in 1981 [25]. Glycophases like LiChrosorb Diol, TSK-SW gels and Separon HEMA 1000 Glc (hydroxyethyl methacrylate polymer covalently bonded with glucose) can be used [26–28].

Of these sorbents with Separon HEMA 1000 Glc [26] yielded the best results. The reason probably is that besides the molecular sieving mechanism other types of interactions between the collagen molecules and the sorbent occur with all the other packings. In actual separations 500×8-mm columns packed with 12–17-μm particles were used. Elution was done with 0.05 *M* Tris–HCl, pH 7.5. made 2 *M* with respect to urea. There is one point to be emphasized with nearly all separations of collagens: triple helical (non-denaturated or denatured) entities occur in the resulting chromatographic recordings side-by-side

with constituting α chains. Typically in a Separon HEMA run the elution sequence of individual constituents of the mixture is α_1(I), α_2(I), and a trimer containing two α_1 and one α_2 chain (called the γ fraction). No β fraction (dimers of the parent α chains) could be distinguished (reportedly they co-elute with the trimers). On the other hand if Separon HEMA 1000 Glc is used as the column packing it is possible to distinguish collagen type I trimers from the disulphidic bonds possessing trimers of collagen type III (Fig. 7). If the sample of collagen type III is treated with performic acid by which operations the disulphide bonds are oxidized and split it is possible to obtain incompletely separated α_1 and α_2 peaks of collagen type I, and also a clearly separated peak of α_1(III).

However, there is still another problem with separations carried out with Separon HEMA 1000

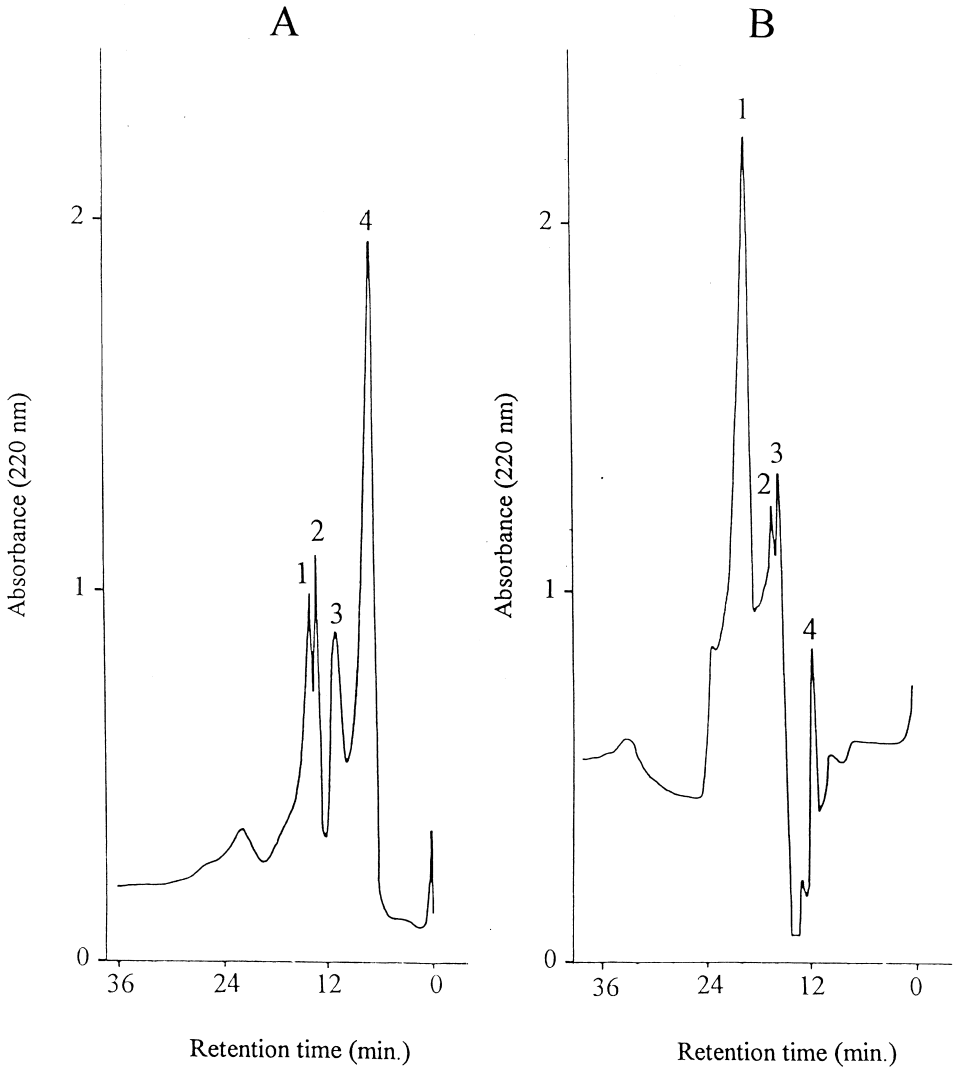

Fig. 7. Separation of collagen chains present in type III before (A) and after (B) performic acid treatment. Conditions: column, Separon HEMA 1000 Glc (50×0.8 cm); elution isocratic with 0.05 *M* Tris–HCl, pH 7.5, containing 2 *M* urea. Peak identification (A) 1, α_1(I); 2, α_2(I); 3, $[\alpha_1(I)]_2\alpha_2(I)$+chain dimers; 4, higher polymers. (B) 1, α_1(III); 2, α_1(I); 3, $[\alpha_1(I)]_2\alpha_2(I)$+chain dimers of type I collagen. From Ref. [26] with permission, modified.

Glc, namely the formation of negative peaks that appear if 0.05 *M* Tris–HCl, pH 7.5, is used as the eluting buffer. This drawback can be overcome by making the mobile phase 0.2 *M* with respect to NaCl. Moreover addition of NaCl to the mobile phase allows the separation of collagen type I chain dimers, however, the separation takes a bit longer. As a matter of fact Separon HEMA can be exploited for the separation of a number of collagens and collagen fragments as shown in Table 2 [29]. All these separations are directed to preparative (semi- and micropreparative) purposes. Their analytical applicability is far superseded by other separation techniques, mainly by polyacrylamide gel electrophoresis. The preparative applications possess some limitations: not all the entities involved could be baseline separated and, consequently, contaminated preparations are frequently obtained.

If the separation with Separon HEMA 1000 Glc is run in the presence of NaCl in the mobile phase, individual collagenous analytes are eluted in the order of increasing relative molecular mass. This relation is not strictly linear in the semilogarithmic plot. On the other hand if the separation is run in the absence of salt in the eluant, a partial separation of, e.g., α_1(I), α_2(I) and α_1(III) can be obtained. In other words the ionic strength of the eluting buffer is of decisive importance for the result.

It was concluded that the above-described separations are ruled by a mixed mode mechanism, in particular by the hydrodynamic volume, adsorption/partition interactions and affinity of the separated collagenous species to the sorbent used. It is evident that, depending on the nature of collagen molecule (chain, fragment) to be separated it is inevitable to combine at least two preparative chromatographic steps. An idea about which types of chromatography can be combined is presented in Fig. 8 [14,20,24,30–47].

Size-exclusion chromatography on rigid gels (PL-

Table 2
Retention time of different collagen chains separated by HPLC on Sepharon HEMA 1000 Glc column (50×0.8 cm); elution: isocratic with 0.2–2 *M* urea–0.05 *M* Tris–HCl, pH 7.5 buffer

Type of collagen chain	M_r	Retention time (min)	Principle of separation
α_2(I), α_1(II)	100 000	42.0[a]	Rat skin, cartilage and calf skin, limited pepsin digestion
α_1(IV)	140 000	38.5	Mouse tumor and human placenta, limited pepsin digestion
α_2(IV)	160 000	37.0	
β(I)	200 000	34.0	Human placenta, limited pepsin digestion
γ(I)	300 000	14.0	
γ(III)	300 000	14.0	Skin
α_1(IV) BM	160 000	37.0	EHS tumor, limited pepsin digestion
α_2(IV) BM	180 000	34.5	
α_{1-3}(V)	110 000	41.5	Human placenta, limited pepsin digestion
C_1 fragment	120 000 (110 000 to 140 000)	41.0[c]	Minces of whole placental tissues, limited pepsin digestion
C fragment	95 000	43.0	
50 K fragment	50 000	50.0[b]	
7 S	360 000	4.0	Mouse tumor basement membrane, limited pepsin digestion
7 S coll	225 000	29.0	

The α_1(I) served as internal standard (according to Deyl et al. [29]). From Ref. [30] with permission.
[a] These collagen types yield repeatedly differing retention times when run in the pure form. In a mixture only a single peak is observed.
[b] This fragment is always fused with the 100 000 band of α-chains, see Footnote a.
[c] Position of this fragment varies.

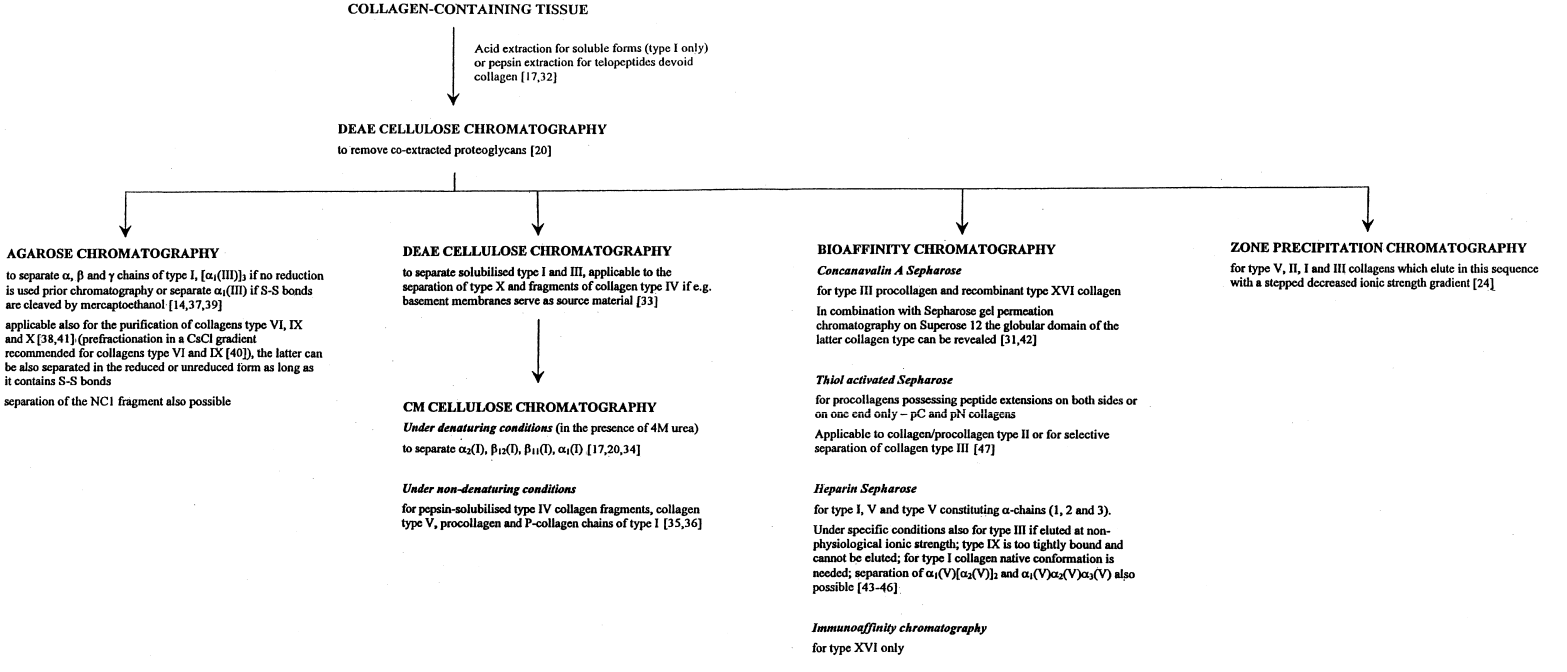

Fig. 8. Fundamental strategy about the application of different chromatographic methods for the separation of collagen types. See also Refs. [14,20,24,31–47]. From Ref. [30] with permission.

Aquagel-OH columns) has been recently used for separation of collagen chain polymer and fragments present in two types of commercial gelatin (obtained from alkali-treated bones and pharmaceutical/food-grade type obtained from acid-treated fish skin) [48]. Though explicitly said in the quoted paper the types of collagen involved were type I and III.

The columns used were 30×0.75 cm in size, with 8-μm sized particles connected in series with a guard column. The mobile phase used was 18 g/l SDS in Milli-Q water passed through the bed at 0.5 ml/min. The dominant peak refers to α-chains, further to the right are degradation products, on its left hand side the chain polymers.

2.3. Classical preparative procedures for parent α-chains and their dimers

For collagen types I and III the classical method offering separation of the individual parent chains and their dimers is carboxymethyl (CM)-cellulose chromatography [49].

Collagen $[\alpha_1(III)]_3$ peak is located between peaks of β_{11} and β_{12}. In practice a 1.5×8.5-cm column is used. The column is maintained at 40 °C, equilibrated with 0.01 *M* potassium acetate–1 *M* urea buffer at pH 4.8 prior to operation. The column is eluted with a linear gradient of 0–0.14 *M* LiCl in 500 ml of buffer [49]. Alternatively sodium acetate buffer can be used instead. In this case the gradient is generated by using 0.06–0.16 *M* buffer. The concentration of the limiting buffer is adjusted by making the solution 0.1 *M* with respect to NaCl. Two 400-ml vessels are used to generate the gradient, an 18×2.2-cm column was recommended for this type of separation [18,50].

For the ways of separating parent polypeptide chains of other collagenous types (as well as their collagenous and noncollagenous domains) the reader should be directed to the respective sections of this review dealing with the particular collagen type.

As a lot effort in this direction is done by using fibroblast cultures, the isolated quantities (if isolated at all) are very small and polyacrylamide gel electrophoresis is the method of choice for both analytical and preparative purposes (see Section 20).

3. Type IV collagen

Type IV collagen is highly polymerized and serves as a framework for endothelial cells and as a selective barrier between blood and the surrounding tissue [51]. As a matter of fact it represents the main component of basement membranes.

Up to now at least six distinct collagen type IV α-chains were described. The classic version of this collagen type is composed of α_1(IV) and α_2(IV) chains. The novel α_3(IV) to α_6(IV) chains can be found only in specific basement membrane types [52].

The classic type IV collagen heterotrimer $[\alpha_1(IV)_2\alpha_2(IV)]$ contains a carboxy terminal NC1 (non-collagenous) domain and a relatively long triple-helical domain of 400 nm with several small interruptions allowing flexibility to the helical rod. The molecules have the property to self-aggregate into dimers by association of their carboxy-terminal (NC1) domain and tetramers by association of their amino-terminal (7S) domains [53,54].

Owing to the pronounced polymerization (insolubility) of collagen type IV its parent α chains could be obtained from animals in which the in vivo crosslinking is blocked (lathyritic animals). Moreover it is necessary to have an animal model in which basement membranes are intensively synthesized; therefore EHS (Ehler–Danlos syndrome) sarcoma serves as source material. From this tumor two types of collagen type IV α chains M_r 160 000 and 140 000 [α_1(IV), α_2(IV), respectively] can be isolated. Their preparation is based on stepwise addition of NaCl to the extract [27].

All extraction procedures are carried out at 4 °C in the presence of protease inhibitors: 2 m*M* *N*-ethylmaleimide and 8 m*M* ethylenediaminetetraacetic acid. Harvested tumor tissue is homogenized in 3.4 *M* NaCl, 0.05 *M* Tris–HCl buffer, pH 7.4, containing the above concentration of protease inhibitors. The extraction can be done with different buffers, for instance: (1) 0.5 *M* NaCl, 0.05 *M* Tris–HCl, pH 7.4; (2) 2.0 *M* guanidine–HCl, 0.05 *M* Tris–HCl, pH 7.4; (3) 2.0 *M* guanidine–HCl, 0.05 *M* Tris–HCl, pH 7.4, containing 2 m*M* dithiothreitol (DTT); and (4) 4.0 *M* guanidine–HCl, 0.05 *M* Tris–HCl, pH 7.4, containing 2 m*M* DTT. The highest

yields of the collagenous material are obtained with DTT-containing buffers. The isolated material is generally >95% pure (judged by the pattern of proteins apparent after gel electrophoresis on 5% gel).

Collagen type IV can be further purified by DEAE-cellulose column chromatography. For instance, the guanidine–DTT extract is dialyzed against 4 *M* urea, 0.05 *M* Tris–HCl, pH 7.4, containing 0.25 *M* NaCl and 2 *M* DTT and chromatographed on a DEAE-cellulose column equilibrated with the same buffer. The unbound material from the chromatographic separation is dialyzed against 4 *M* urea, 0.05 *M* Tris–HCl, pH 7.4, containing 2 *M* DTT and rechromatographed under the same conditions as above [55].

3.1. NC 1 (non-collagenous) component of collagen type IV

Adult bovine aorta or glomerular basement membrane serves usually a source tissue for this component.

The tissue (85 g) is cut into small pieces and extracted at 4 °C for 24 h in 0.5 *M* KCl, 0.1 *M* Tris–HCl, pH 7.5, containing protease inhibitors (1 m*M* phenylmethanesulfonyl fluoride, 10 m*M* EDTA, 25 m*M* 6-aminohexanoic acid, 4 m*M* *N*-ethylmaleimide and 5 m*M* benzamidine–HCl). The ground tissue is next suspended in 500 ml 6 *M* guanidine–HCl, 50 m*M* Tris–HCl, pH 7.5, stirred for 36 h at room temperature and centrifuged. The supernatant is discarded and the pellet is washed four times with deionized water and twice with collagenase buffer (50 m*M* Tris–HCl, pH 7.5, 2 m*M* $CaCl_2$, 4 m*M* *N*-ethylmaleimide, 1 m*M* phenylmethanesulfonyl fluoride). Next the pellet is resuspended in 200 ml collagenase buffer at 37 °C, bacterial collagenase is added and the digestion is allowed to proceed for 36 h with gentle stirring. After this period digestion is stopped by adding EDTA to the final concentration of 30 m*M*. The digestion mixture is centrifuged and the NC1 domain hexamer is isolated from the supernatant by DEAE-cellulose chromatography followed by size-exclusion chromatography on a Sephacryl S-300 column (35×2.5 cm), equilibrated again with 50 m*M* Tris–HCl, pH 7.5, which serves simultaneously as mobile phase. This procedure serves the separation of NC1 domain from the 7S domain of collagen type IV [51].

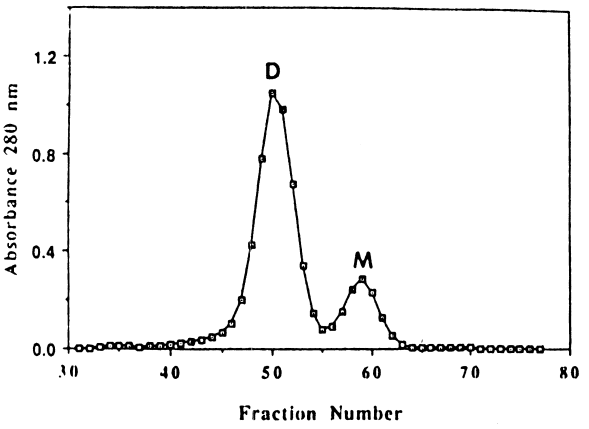

Fig. 9. Isolation of monomeric and dimeric components of the NC1 domain hexamer. The purified NC1 hexamer is concentrated, heated in 6 *M* guanidine–HCl at 85 °C and run through a Sephacryl S-200 column at room temperature. D, dimer; and M, monomer. The dimer–monomer ratio is 77:23; an essentially identical value is obtained from a spectrophotometric scan of an SDS–PAGE gel. From Ref. [56] with permission.

Separation of monomeric and dimeric components of the NC1 hexamer can be done as follows. The purified hexamer after Sephacryl S-300 gel permeation chromatography is concentrated by an ultrafiltration apparatus equipped with a YM10 filter, dialyzed against 6 *M* guanidine–HCl, 50 m*M* Tris–HCl, pH 7.5, for 24 h. The dialyzed sample is heated to 85 °C for 10 min and passed through a Sephacryl S-200 column (90×2.5 cm) using 6 *M* guanidine–HCl, 50 m*M* Tris–HCl, pH 7.5 as mobile phase. The subunits of the NC1 hexamer are thus resolved into two peaks, corresponding to the dimeric and monomeric components, respectively (Fig. 9) [56].

4. Type V collagen

Type V collagen is a quantitatively minor fibrillar collagen with a broad tissue distribution. The most common type V collagen isoform is $[\alpha_1(V)_2\alpha_2(V)]$ found in cornea. However, other isoforms exist, including $[\alpha_1(V)\alpha_2(V)\alpha_3(V)]$ form, an $\alpha_1(V)_3$ homotrimer and a hybrid of type V/XI α-chains. The

functional role and fibrillar organization of these isoforms is not yet fully understood [57].

Type V collagen can be purified from fetal bovine cortical bone. Bone fragments are demineralized in 0.5 *M* EDTA, 0.05 *M* Tris–HCl, pH 7.5, containing protease inhibitors (2 m*M* phenylmethylsulfonyl fluoride, 5 m*M* *N*-ethylmaleimide and 5 m*M* benzamidine–HCl) at 4 °C for 1 week. After powdering in a liquid N_2 mill, the bone is treated with pepsin at 1:10 (w/w/) in 0.5 *M* acetic acid at 4 °C for 24 h. The pepsin digest is clarified by centrifugation and collagens are fractionated by sequential precipitation at 0.8 *M* and 1.2 *M* NaCl [58].

4.1. Separation of α-chains collagen type V from collagen type XI

Separation of collagen type V $\alpha_1+\alpha_2$ chains from the α_1(XI) polypeptide chain can be done by reversed-phase HPLC (C_8 RP300 column, 25×0.46 cm) by applying a gradient (from 21 to 33%) of acetonitrile–*n*-propanol (3:1, v/v) and water in 0.1% (v/v) trifluoroacetic acid. In this system the α_1(XI) chain is resolved, α_1(V) and α_2(V) coelute (see Fig. 10) [59]. The latter two chains can be separated by a subsequent ion-exchange HPLC step (see Fig. 11) [58]. For this purpose a Bio-Gel-TSK DEAE-5-PW column (7.5×0.75 cm) was used. Samples were denaturated by heating in 6 *M* urea and eluted with a gradient from 0.01 to 0.15 *M* NaCl in 0.02 *M* Tris–HCl, 5% (v/v) 1-propanol, pH 7.5, over 30 min at 1 ml/min. [No identification of the α_3(V) chain was reported in the quoted paper.]

4.2. Different collagen type V α-chains and their trimers

The isolation of different types of collagen type V α-chains from bovine crude type V collagen preparations can be done as follows. The first step is chromatography on Fractogel EMD SO_3^- 650(S) column with 0.04 *M* Tris–HCl buffer pH 8.2 containing 2 *M* urea and 0.05 *M* NaCl with a linear NaCl gradient to the final concentration of 1 *M*. This approach results in four distinct peaks; the last two contain collagen type V α-chains (and possibly their fragments) polymerized by disulfide bonds.

The constituting α(V) chains of the

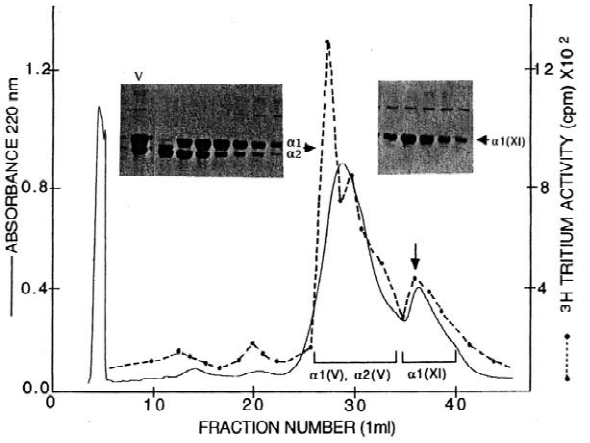

Fig. 10. Reversed-phase HPLC resolution of type V collagen α chains. Purified type V collagen extracted from fetal calf bone by pepsin is treated with NaB^3H_4 and eluted from a C_8 column by an acetonitrile gradient in 0.1% (v/v) trifluoroacetic acid. Aliquots of fractions under the regions of eluate marked by brackets are analyzed by SDS–PAGE (7.5% acrylamide). The α_1(V) and α_2(V) coelute on this column (fractions 25–35; left gel). The α_1(XI) chain is resolved (fractions 36–45; right gel). A sample of the protein loaded on the column is run in gel lane V. From Ref. [59] with permission.

$[\alpha_1(V)\alpha_2(V)\alpha_3(V)]$ and $[\alpha_1(V)]_2\alpha_2(V)$ can be resolved from the crude preparations on Bakerbond PEI anion exchanger; higher polymers are hidden in the tailing shoulder of the α_1(V) peak. 0.2 *M* Tris–HCl buffer, pH 7.0, containing 2 *M* urea is used for

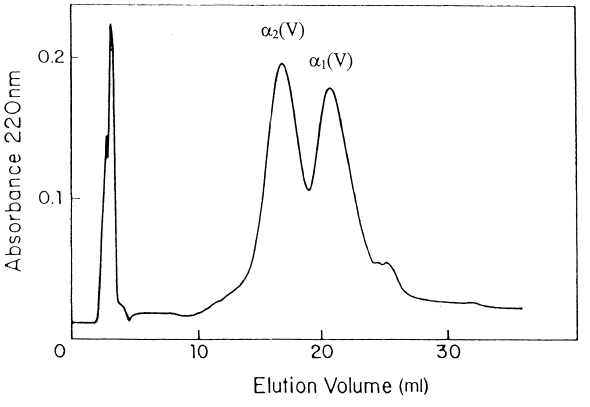

Fig. 11. Anion exchange HPLC of the pooled fractions 25–29 obtained by reversed-phase HPLC Bio-Gel-TSK DEAE-5-PW column. This procedure leads to a clear-cut separation of α_1(V) and α_2(V) polypeptide chains. From Ref. [58] with permission, modified.

sample application and the elution is effected by an NaCl gradient from 0 to 35 *M* over 20 min [60] (Fig. 12). The fractions obtained from the former separation step are heated to 50 °C for 5 min before being applied to the Bakerbond PEI column.

5. Type VI collagen

Type VI collagen is another abundant collagenous components present in a number of organs and tissues. It was revealed that the molecule consists of collagenous as well as non-collagenous domains. To isolate this protein is extremely difficult as it is highly insoluble under non-denaturing conditions (it contains disulphide cross-links and appears heavily glycosylated). Consequently most studies were done with the pepsinized material which resulted in the distinguishing three types of collagenous polypeptide chains named α_1, α_2 and α_3, all M_r 140 000 and comigrate on polyacrylamide gel. This holds for the mammalian type VI; in birds (chicken gizzard type VI), however, Schreier et al. were able to distinguish three subunits of M_r 130 000, 140 000 and 180 000–200 000, the largest of which appears composed of three different subspecies that are believed to represent a single collagen subunit processed (glycosylated) posttranslationally to a different extent [61].

The general scheme for collagen type VI (and unmodified constituting α-chains) preparation runs as follows: The starting tissue (100 g) is extracted successively with: (i) 150 m*M* NaCl, 50 m*M* Tris–HCl, pH 7.5; (ii) 1 *M* NaCl, 50 m*M* Tris–HCl, pH 7.5; (iii) 2% (w/v) SDS, 50 m*M* Tris–HCl, pH 7.5. Each buffer must contain a set of protease inhibitors (1 m*M* *N*-ethylmaleimide, 1 m*M* phenylmethanesulfonyl fluoride and 5 m*M* EDTA). The SDS extract is concentrated by ultrafiltration (PM 10, Amicon), dialyzed against 100 m*M* NaCl, 0.2% SDS, 1 m*M* EDTA, 50 m*M* Tris–HCl, pH 7.5, and denaturated by heating to 80 °C for 5 min. After centrifugation the solubilized protein is purified on a Sephacryl S-500 column (Pharmacia 70×3.3 cm, 17.4 ml/h) at room temperature. The complex profile obtained is divided into aliquots, individual fractions are reduced with mercaptoethanol, alkylated with 4-vinylpyridine and chromatographed on a S-400 Sephacryl column (67×2.5 cm, 13.7 ml/h).

It is noticeable that chromatographic separation of the intact α_2(VI) (M_r 130 000) and α_1(VI) (M_r 140 000) on a preparative scale is not possible owing to their extremely close properties in terms of charge and hydrophobicity (attempts to use CM-cellulose, DEAE-cellulose or reversed-phase chromatography failed). The only way how to overcome this difficulty is to use preparative gel electrophoresis as demonstrated in the quoted paper of Schreier et al. [61].

Alternatively it is possible to prepare pepsinized collagen type VI. This way of type VI collagen solubilization reveals upon polyacrylamide gel three major bands of M_r 35 000, 45 000 and 55 000

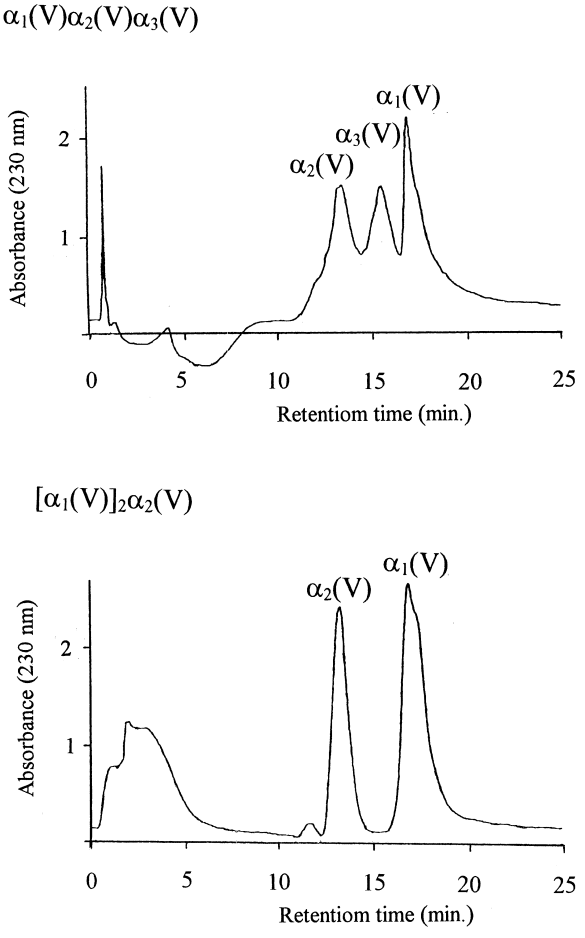

Fig. 12. Separation of subunit chain from human α_1(V)α_2(V)α_3(V) and [α_1(V)]$_2\alpha_2$(V) molecules by Bakerbond PEI Scout column (From Ref. [60] with permission, modified).

subunits (representing α_1, α_2 and α_3 parent peptides devoid of non-collagenous domains). The preparative procedure for obtaining pepsinized type VI collagen runs as follows. Prewashed human placenta is digested with pepsin by two different procedures (100 mg of enzyme per kg of wet mass). In the first procedure the placenta is homogenized in 0.5 *M* formic acid, pH 2.5 (1.5 l/kg), and incubated with pepsin for 24 h at room temperature. In the second method the homogenization is in 0.5 *M* acetic acid, pH 2.9, and the incubation with pepsin is for 24 h at 6–8 °C. Interstitial and basement-membrane collagens are removed from both digests by precipitation with 1.2 *M* NaCl for 24 h at 6–8 °C. The NaCl concentration of the supernatants is then raised to 1.7 *M*, which precipitates intima collagen together with some non-collagenous proteins [62]. The precipitate is dissolved in 0.1 *M* acetic acid and dialysed at 6–8 °C against a large volume of 20 m*M* sodium phosphate buffer, pH 7.2. The precipitated protein is once more subjected to fraction NaCl precipitation and purification by dialysis. The final precipitate is then dissolved in 0.1 *M* acetic acid (about 5 mg/ml) and dialysed against 1 *M* $CaCl_2$, 50 m*M* Tris–HCl buffer, pH 7.4, and a volume of the solution containing about 100 mg of protein is then passed over an agarose Bio-Gel A-5m column (130×3 cm), equilibrated with the same buffer. Intima collagen emerges as a major fraction close to the void volume of the column, and this fraction is dialyzed against dilute acetic acid and freeze-dried [63].

Dimers and tetramers of the α chains of collagen type VI could be isolated as follows: 200 g of amniotic membranes of human placenta is homogenized in the 50 m*M* Tris–HCl buffer, pH 7.0, containing 1 *M* NaCl. The tissue residue is collected by centrifugation and washed with the same buffer once more, followed by two washes with 0.5 *M* acetic acid. The insoluble residue is extracted overnight with 3 vol (v/w) of 0.1 *M* Tris–HCl buffer, pH 7.8, containing 5.5 *M* guanidine–HCl (final concentration) and centrifuged at 18 000 *g* for 1 h. The supernatant is next dialyzed extensively against 1% acetic acid. The precipitate formed during dialysis is collected by centrifugation and redissolved in 40 volumes (v/w) of the buffer described above but containing 6 *M* guanidine–HCl. The solution is clarified by centrifugation, and the supernatant solution is again dialyzed against 1% acetic acid. The precipitate formed during dialysis is collected by centrifugation and dissolved in 40 vol (v/w) of 0.05 *M* Tris–HCl containing 6 *M* urea and 0.12 *M* NaCl, pH 8.3 (DEAE buffer), dialyzed against 2 l of the same buffer, and clarified by centrifugation. DEAE-cellulose, which has been equilibrated with the same buffer, is stirred into the supernatant solution. Proteins which did not bind to DEAE-cellulose are collected, concentrated with Bio-Gel concentrator resin, and chromatographed on a Sephacryl S-500 column (80×5 cm) equilibrated at room temperature with 0.04 *M* Tris–HCl buffer, pH 6.8, containing 6 *M* urea and 0.1 *M* Na_2SO_4 (TSK buffer). Column fractions which contain type VI collagen identified by monoclonal antibody, gel electrophoresis, and their electron micrographs after rotary shadowing, are pooled, dialyzed against 1% acetic acid, lyophilized, and stored at −80 °C. Parent collagen type VI domains can be obtained from this preparation after reduction. The final yield of type VI collagen is approximately 0.5% (w/w) of acid-extracted tissue residue. Depending upon the requirements of experiments, type VI collagen can be further purified by using two Bio-Sil TSK-400 columns (60×2.15 cm) connected in tandem and equilibrated with TSK buffer [64]. Amino-terminal and carboxy-terminal non-helical fragments (GRBC peptides) of collagen type VI can be prepared from guanidine-hydrochloride-extracted tetramers that are reduced, alkylated and treated with bacterial collagenase as described in the paper of Jander et al. [65]. Calf aorta or ligamentum nuchae is homogenized and extracted successively with solutions of 0.9% saline (extract S), 0.5 *M* KCl (extract SK), 6 *M* guanidine hydrochloride (extract SKG), and 6 *M* guanidine hydrochloride with 0.05 *M* dithioerythritol (extract SKGD) [66]. Each extraction solution also contains a cocktail of protease inhibitors and is buffered to pH 7.5 with 0.05 *M* Tris–HCl. The extractions are allowed to proceed at 4 °C for 24 h. The lyophilized extracts SKG and SKGD of calf aorta are extracted further with 0.05 *M* Tris, pH 7.5, at 37 °C, and both supernatants and residues are reduced with mercaptoethanol and alkylated with vinylpyridine. Ion-exchange chromatography is carried out on DEAE-

cellulose (DE-52, Whatman) in 6 *M* urea and 0.05 *M* Tris, pH 8.3, at 20 °C [65,67].

Alternatively the globular domains of type VI collagen can be isolated also from bacterial collagenase-digested monomers [65]. Purified bacterial collagenase (4 ml at 0.1 mg/ml) is added to 200 ml monomer solution (0.2 mg/ml) in buffer containing protease inhibitors plus 0.4 *M* NaCl. After 2 h incubation at 55 °C, a fresh aliquot of enzyme and a freshly prepared mixture of protease inhibitors is added. A third aliquot of enzyme and inhibitors is added and the digestion continued at 45 °C for another 2 h and, after a fourth addition of enzyme and inhibitors, incubation is continued at 37 °C overnight. The degradation products of the helix and salt are removed by extensive dialysis against 1% acetic acid and the dialysate is lyophilized. Native globular domains can be finally purified on a Sephacryl S-500 column (110×1.5 cm) equilibrated with 50 m*M* Tris–HCl, pH 7.0, containing 0.1 *M* NaCl and 0.05% octylglucoside [68].

6. Type VII collagen

This collagen type represents the major component of anchoring fibrils in skin basement membrane. Using cDNA cloning and amino acid analysis it was revealed that the protein is formed by three identical α-chains, each component of M_r 145 000 central collagenous domain with several interrupting non-collagenous sequences. The collagenous domain is flanked by an amino terminal non-collagenous domain (NC1) and a smaller C-terminal non-collagenous domain (NC2) of M_r 35 000. In tissues this protein forms antiparallel tail-to-tail dimers with a small carboxy-terminal overlap. During maturation most of the NC2 domain is proteolytically removed [69].

Type VII collagen is prepared from the human cell line, KB, originally derived from an oral epidermoid carcinoma [70]. Proteins secreted from confluent monolayers are collected during a 24-h incubation in serum-free medium containing 100 ng/ml ascorbic acid. Following collection, unattached cells are removed by centrifugation and EDTA, *N*-ethylmaleimide, and phenylmethanesulfonyl fluoride are added to final concentrations 5 m*M*, 50 μ*M*, and 50 μ*M*, respectively. Proteins are precipitated by the addition of ammonium sulfate (300 mg/ml) and incubated on ice overnight. The precipitated proteins are next collected by centrifugation (1.2×10^6 *g*) and resuspended in 25 m*M* Tris–HCl, pH 7.8, 65 m*M* NaCl, containing 1 m*M* EDTA. Samples are then treated with diisopropyl fluorophosphate (4 μg/ml) and dialyzed against resuspension buffer. Next the insoluble material is centrifuged (300 000 *g*) and the supernatant (S1) is stored at −20 °C [71]. The pellet of the insoluble material can be re-extracted and purified as described above. The second supernatant arising from this treatment is combined with the first one and subjected to chromatography. For this purpose the sample is re-equilibrated with 0.1 *M* NaCl by dialysis, chromatographed on DEAE-cellulose (Whatman DE-52, 10×1.6 cm), and eluted at 1.0 ml/min with a NaCl gradient using a dual buffer system: 50 m*M* Tris–HCl, pH 7.8, and 2 *M* urea (buffer A) and 50 m*M* Tris–HCl, pH 7.8, 2 *M* urea, and 1 *M* NaCl (buffer B). Following a 10-min wash with 10% B, the retained proteins are eluted first with a 20-min linear gradient from 10 to 50% B and then a 10-min linear gradient from 50 to 100% B is introduced which elutes type VII procollagen (Fig. 13) [72].

Extraction of collagen type VII is also possible by using 6 *M* guanidine hydrochloride and amniotic membranes as source tissue [73].

As with other collagen types, a further insight in the collagen type VII structure can be obtained by pepsin treatment which splits the molecule at a pepsin-sensitive interruption nearly midway along the triple helix. Separation of these fragments (called P_1 and P_2) can be done after disulphide bond reduction using C_{18} reversed-phase chromatography [74]. Alternatively these fragments can be prepared also by molecular sieving making use of TSK 4000 column (120×2.15 cm) equilibrated and eluted with 10 m*M* Tris–acetate, pH 6.8, 6 *M* urea, 60 m*M* Na_2SO_4 at 0.5 ml/min (a C_4 sorbent can be used as well).

Separation of codistributed collagen types V and VII can be achieved by CM-cellulose chromatography as described by Bentz and co-workers (Fig. 14) [73,74].

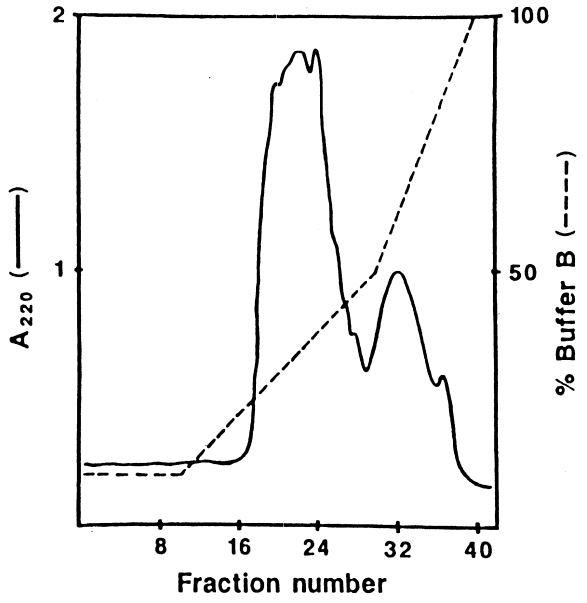

Fig. 13. HPLC-DEAE chromatography of S1 supernatant proteins. The absorbance profile for proteins eluted from HPLC-DEAE column.Type VII collagen is eluted in fractions 28 and 29. From Ref. [72] with permission. The dashed line refers to the proportion of buffer B in the mobile phase.

Collagen VII can be co-isolated with type V collagen from human amnions. The amniotic membranes are separated and soaked in distilled water. The homogenates are solubilized by limited pepsin digestion [0.05 g per 100 g (wet mass) of amnion], and the solubilized collagen can be precipitated from the clarified supernatant by addition of NaCl to a concentration 1 *M*. The precipitate is dissolved in cold 1 *M* NaCl, 50 m*M* Tris–HCl at 7.5, titrated to pH 8.6 to inactivate pepsin, and reprecipitated by exhaustive dialysis against 0.01 *M* Na_2HPO_4. The recovered precipitate is redissolved in, and dialyzed against 0.5 *M* acetic acid [75]. Under non-denaturating conditions collagen type VII can be separated from type V by chromatography on CM-cellulose (CM-52, Whatman; 16×2.5-cm column) equilibrated with 50 m*M* LiOAc, pH 4.8, 2 *M* urea at room temperature. The column is developed with a superimposed 1200-ml linear gradient from 0 to 0.3 *M* LiCl. Denaturated α(VII) chains are purified by an additional chromatographic step on DEAE-cellulose (DE-52, Whatman; 16×1.6 cm, equilibrated with 45 m*M* Tris–HCl, pH 8.6, 4 *M* urea, containing 100 μl of thiodiglycol per liter and maintained at 42 °C). The column is developed with a superimposed 800-ml linear gradient from 0 to 0.16 *M* NaCl [73].

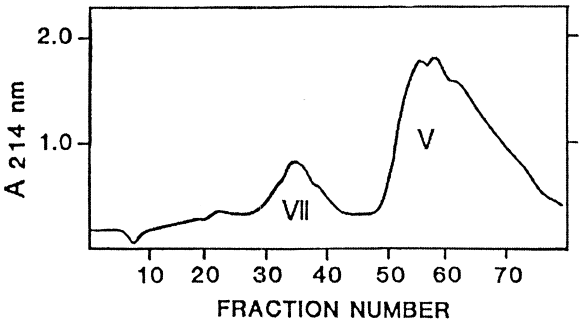

Fig. 14. Separation of types V and VII collagens. A mixture of types V and VII collagens obtained by salt precipitation can be resolved by chromatography on CM-cellulose under non-denaturating conditions. From Ref. [74] with permission, modified.

7. Type VIII collagen

Type VIII collagen is usually prepared from Descement membrane where it is co-distributed with collagen type X.

The tissue is subjected to limited pepsin digestion and differential salt precipitation. Type VIII collagen (M_r 50 000, triple helical fragment) can be obtained by gel permeation chromatography of the mixture resulting from enzymatic digestion as follows: bovine corneal endothelial cells are cultured in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum, supplemented with penicillin–streptomycin. Metabolic labeling of cells is carried out on nearly confluent cultures. The labeling medium contains no serum and is supplemented with 50 μg/ml sodium ascorbate, 64 μg/ml β-APN, and 25 μCi/ml L-[2,3-^{3}H]proline (35 Ci/mmol). After 20–24 h incubation with the isotope, the medium is removed into 5 m*M* *N*-ethylmaleimide, 0.2 m*M* $PhCH_2SO_2F$, and 2.5 m*M* EDTA. The medium is centrifuged at 5000 *g* for 5 min to remove cellular debris, and solid ammonium sulfate is added with

constant stirring at 4 °C to a final concentration of 50% (mass to initial volume ratio). Twelve hours later, the precipitate is collected by centrifugation at 20 000 *g* for 30 min at 4 °C. It is redissolved in a 6 *M* urea, 50 m*M* Tris–HCl, 0.2 m*M* $PhCH_2SO_2F$, and 2.5 m*M* EDTA buffer, pH 8.0, dialyzed against the same buffer at 4 °C (with at least three buffer changes), and chromatographed on DEAE-cellulose at 4 °C [76]. The unbound proteins from the DEAE-cellulose column are dialyzed against 0.5 *M* acetic acid at 4 °C. The dialysate is digested with pepsin (100 μg/ml) in a dialysis bag for 2 h at 4 °C followed by lyophilization [77].

In order to separate the subunits, the purified preparations are heated to 60 °C for 30 min in 6 *M* guanidine, 0.1 *M* Tris, pH 8.5; next the mixture is acidified with trifluoroacetic acid to pH 2 and subjected to reversed-phase chromatography (Vydac C_{18}, 25×0.46-cm column) using water with 0.1% trifluoroacetic acid as solvent A and 0.1% trifluoroacetic acid in acetonitrile–water (70:30, v/v) as solvent B (from 0 to 30% B over 10 min followed by 30 to 50% B over the next 80 min, flow-rate 0.5 ml/min). HPLC separation yields occasionally a double peak of the α_1(VIII) as well as a double peak of α_2(VIII) (Fig. 15). The reason for the existence of these double peaks during reversed-phase chromatography is not known; on conventional gel electrophoresis this effect has not been observed. On the other hand if the reversed-phase chromatographic step is performed with W-Porex reversed-phase column using a linear gradient of 20–40% (v/v) acetonitrile–water with 0.1% trifluoroacetic acid, the above-mentioned doubling of the α_1(VIII) and α_2(VIII) peaks was not observed (Fig. 16) [78].

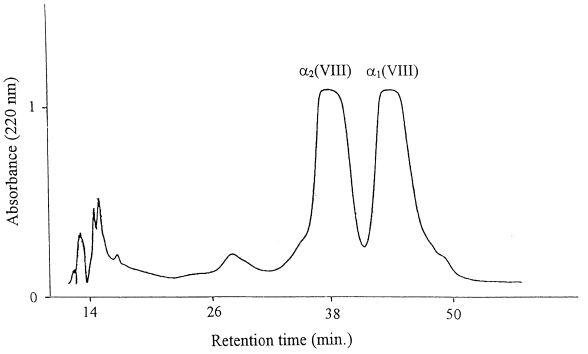

Fig. 16. Reversed-phase HPLC separation of the α-chains of bovine Descement's membrane type VIII collagen. Chromatography of Descement's membrane type VIII collagen on W-Porex reversed-phase column using a linear gradient of 20–40% in acetonitrile–water (v/v) with 0.1% trifluoroacetic acid. From Ref. [78] with permission.

Regarding stoichiometry of the collagen type VIII constituting α-chains it appears feasible to conclude that collagen type VIII is formed by two molecular entities namely $[\alpha_1(\text{VIII})]_3$ and $[\alpha_2(\text{VIII})]_3$ [79].

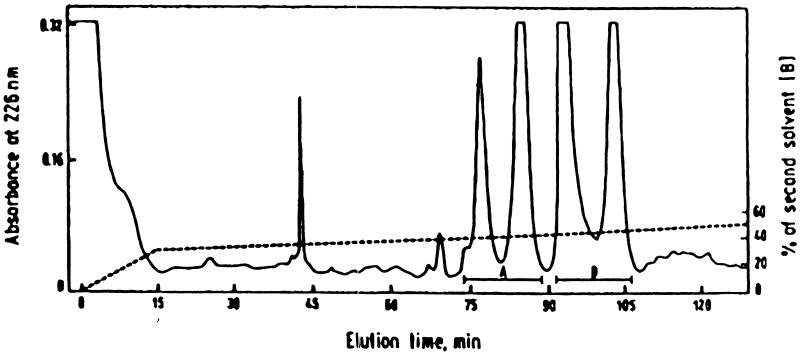

Fig. 15. HPLC separation of α_1(VIII) and α_2(VIII) chains after pepsin extraction and denaturation. The products are identified by N-terminal sequencing. The two peaks denoted "A" contained the pure triple-helical region of the α_2 chain, the two peaks denoted "B" contained the pure triple-helical region of the $\alpha_1$chain. The reason for the splitting into double peaks is not known. Gel electrophoresis did not shown any difference in mobility (not shown). Higher column temperatures did not change this peak pattern. From Ref. [79] with permission.

8. Type IX collagen

Collagen type IX (a representative of the FACIT family[1]) can be separated from other cartilage collagens (types II and XI) by hydrophobic interaction chromatography on a. stainless-steel column (25× 0.8 cm) packed with Separon HEMA 1000 Bio. The recommended mobile phase is 0.84 *M* ammonium sulphate made 0.1 *M* with respect to KH_2PO_4 (pH 6.5). Under these conditions only collagen type IX is eluted from the column; it could be monitored with UV detection (218 nm) or selectively with fluorescence detection (excitation 330 nm, emission filter 389 nm). The method can be used both for isolation and quantitation of this collagen type. The analytical assay is reportedly linear in the range 0–10 μg with the detection limit 0.6 μg [26].

9. Type X collagen

Collagen X is a short-chain collagen expressed in the hypertrophic zone of calcifying cartilage during skeletal and bone growth. The α_1(X) homotrimer consists of three distinct protein domains [82].

Collagen type X can be isolated from chick-embryo cartilages. Explants of tibiotarsal and femoral cartilages are found to synthesize type IX collagen in zones 1 and 2, whereas type X collagen was shown to be a product of the hypertrophic chondrocytes in zone 3. Pulse-chase experiments with tibiotarsal (zone-3) explants demonstrated a conversion of type X procollagen into polypeptides of M_r 45 000–49 000. Pro-α_1(X) chains occur as short rod-like molecules 148 nm in length with a terminal globular extension; the processed species comprising α_1(X) chains of M_r 45 000–49 000 represents the linear 148-nm segment devoid of the globular extension [83].

For preparation the cartilage material is crushed in liquid nitrogen, digested with pepsin in 0.1 *M* acetic acid, clarified by centrifugation, precipitated stepwise at 0.8, 1.2 and 2.0 *M* NaCl. Collagen type X precipitates at 2.0 *M* NaCl; it is redissolved by dialysis against 0.1 *M* acetic acid separated from high-molecular mass components by CM cellulose chromatography using a linear 0–0.2 *M* NaCl gradient [83].

Alternatively the culture medium can be treated with ammonium sulphate to 30% concentration and the precipitate collected by centrifugation and redissolved in phosphate buffer, pH 7.6. After a second precipitation step the product is treated with pepsin and subjected to gel permeation chromatography on a 98×0.9-cm Sephacryl column (thermostated to 8 °C). For further purification 5×1.5-cm CM-cellulose column thermostated to 42 °C and eluted with a linear gradient NaCl 0–16 *M* in 0.02 *M* acetate, pH 4.8. in this case CM-cellulose chromatography should be done at denaturing conditions (Fig. 17) [84].

10. Type XI collagen

Collagen XI is a relatively minor fibrillar collagen that regulates the assembly of cartilaginous matrices by copolymerizing with collagen II trimers [85]. Collagen XI consists of three subunits, one of which (α_3 chain) corresponds to the unprocessed form of α_1(II) collagen [31]. Evidence has rapidly accumulated to indicate a broader than previously thought expression of collagen XI in cartilaginous and non-cartilaginous tissues [58]. Subsequent analyses revealed the existence of α_1(XI) and α_2(XI) collagen

[1]FACIT collagens (type IX, XII, XIV, XVI, XIX, XX, XXI and XXV).
The molecules of FACIT (fibril-associated collagens with interrupted triple helices) subfamily are characteristic in possessing a number of collagenous (CO) domains interrupted by short non-collagenous (NC) regions [80], however in some cases the terminal domains could be quite extensive. FACIT collagens are found attached to the fibril surface and they are typical in binding to other matrix components.
Regarding the nature of the N-terminal domain all six FACIT collagens possess a thrombospondin-like N-terminal-like domain (TSPN); collagens type XII, XIV, XX and XXI have varying number of von Willebrand factor A (VWFA)-like domains [81].
While the role of TSPN domain remains unclear, it appears that the VWFA domains are responsible for the interaction of these collagen types with other matrix components.
The above set of collagen proteins was discovered using bioinformatic tools and their presence in tissues was documented by means of immunohistochemical techniques in which the antibodies were risen against a typical sequence of the particular collagen. To our best knowledge only scattered information is available so far for these minor collagen types.

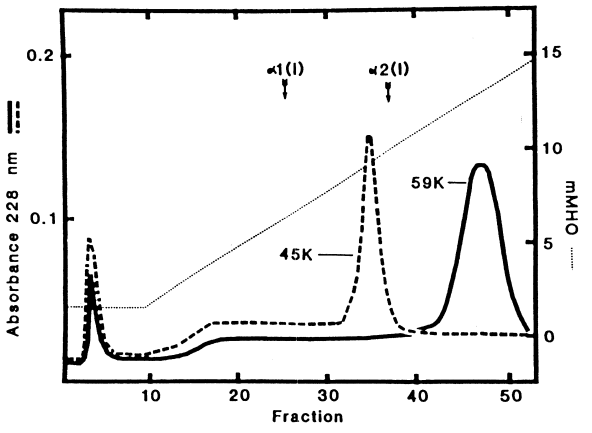

Fig. 17. CM-cellulose chromatograms of the two forms of SC collagen run under denaturating conditions. The elution profile of the M_r 45 000 chains are shown by dashed line; the M_r 59 000 chains are shown by the solid line. The two arrows mark the elution positions of the α_1 and α_2 chains of chick type I collagen. The dotted line represents the conductivity of the fractions collected from the NaCl elution gradient. From Ref. [84] with permission.

isoforms resulting from alternative splicing of the primary transcripts [86,87].

For the separation of this collagen type see Section 4.1. Separation of α-chains of collagen type IV from collagen type XI.

11. Type XII collagen

Type XII collagen, another member of the FACIT family, is generally co-distributed with collagen type I; however, in corneas it can be found along with collagen type II and IX as well; basically it represents a chain trimer; its polypeptide chains contain several distinct sequence domains [domains that are homologous to the domains of von Willebrandt factor, fibronectin type III repeats of the amino-terminal globular domain of cartilage form of collagen chain α_1(IX)]. Three chains form a central globular structure to which finger-like extensions and a thin tail are attached. The tail is sensitive to bacterial collagenase, forms the collagen part of the molecule and is represented by two domains of Gly-X-Y repeats in the carboxyl region of the α_1(XII) chains. The kink observed in the tail is likely to correspond to a short non-triple helical sequence between the two triple-helical domains [88].

Lately it was demonstrated by Kato at al. [89] that collagen type XII is synthesized in bovine cornea in two isoforms. Only the short isoform was observed in skin. The two isoforms result from alternative splicing of a primary transcript resulting in two molecular entities of M_r 340 000 and 210 000. The proportion of non-helical sequences constitute M_r 310 000 and 190 000 sections (consequently the triple helical sections are relatively small). The larger isoform was proven to be synthesized by cells in culture, whereas the shorter form is that found in tissues.

Already in 1992 the preparative procedure for the large collagen type XII-related protein was published by Koch at al. [90]: The oligomeric protein can be purified from culture media conditioned by skin fibroblast from 11-day-old chick embryos [91] or bovine corneas. In the first approach, 5 l conditioned medium are concentrated 30-fold by precipitation with 40% saturated ammonium sulfate and dialyzed against Tris–NaCl (150 m*M* NaCl, 20 m*M* Tris–HCl, pH 7.4). Fibronectin and tenascin are removed [91]. The remaining protein is applied to a 30-ml column of DEAE-Sephadex A-25 (Pharmacia, Uppsala, Sweden). The column is washed with Tris–NaCl and eluted with 0.5 *M* NaCl and 50 m*M* Tris–HCl, pH 7.4. In this fraction, two fuzzy bands of M_r 350 000 and 600 000 are prominent on SDS–PAGE and were followed during further purification. The pooled eluate is dialyzed against Tris–NaCl and loaded onto a 10-ml heparin–agarose column. After washing with Tris–HCl, the bound material is eluted with 0.6 *M* NaCl and 50 m*M* Tris–HCl, pH 7.4. Finally, 2-ml aliquots of the eluate are chromatographed on a Superose-6B column (Pharmacia) in Tris–NaCl. The oligomeric protein complex can be detected shortly after the void volume of the column.

For isolation of collagen type XII from bovine corneas the working protocol runs as follows [89]: bovine corneas are cut into small pieces and homogenized in 0.2 *M* NaCl, 10 m*M* *N*-ethylmaleimide, 0.1 m*M* phenylmethylsulfonyl fluoride and 25 m*M* Tris–HCl, pH 7.8. The suspension is stirred for 5 days and centrifuged at 12 000 *g* for 30 min. The pellet is discarded, and the supernatant is chromatographed on a DEAE-cellulose (Phar-

macia) column (100 ml) that has been equilibrated with the same buffer. Eluted material is dialyzed against 2.7 *M* NaCl and 0.025 *M* Tris–HCl, pH 7.8. The precipitate formed during dialysis is collected by centrifugation (12 000 *g* for 60 min) and dissolved in the extraction buffer.

12. Type XIII collagen

The α_1 chain of type XIII collagen represents a *trans*-membrane collagen species [92] and consists of three collagenous and four noncollagenous domains, and its primary transcript undergoes complex alternative splicing [93].

The complete primary structure of the mouse type XIII collagen chain was determined by cDNA cloning [93]. Western blotting of human tumor HT-1080 cell extract revealed bands of over M_r 180 000. These appeared to represent disulfide-bonded multimeric polypeptide forms that resolved upon reduction into M_r 85 000–95 000 bands that are likely to represent a mixture of splice forms of monomeric type XIII collagen chains. These chains were shown to contain a hydrophobic transmembrane segment [92]. No preparative chromatographic (or electromigration) procedures are, to our best knowledge, available so far.

13. Type XIV collagen

Collagen type XIV (CXIV), a member of the FACIT family of collagens, as well as the related matrix protein undulin, is found in association with collagen fibrils in differentiated mesenchymal tissue [94,95].

The following procedure can be recommended for collagen type XIV preparation. All separation steps are carried out at 4 °C. Fresh placenta is washed and homogenized in a warning blender. After washing the tissue is extracted for 60 h with 3 l of a solution containing 20 m*M* Tris–HCl, 10 m*M* EDTA, 10 m*M* sodium azide, 1 *M* NaCl, 0.3 m*M* PMSF, 10 m*M* *N*-ethylmaleimide, pH 7.4. After centrifugation at 35 000 *g* for 30 min, the NaCl concentration of the supernatant is adjusted to 5 *M* [96]. Precipitated material is pelleted at 35 000 g for 30 min, then redissolved in the extraction buffer and dialyzed against the same buffer containing 0.1 *M* NaCl but without *N*-ethylmaleimide. The material is passed over a heparin–Sepharose column (80 ml), and the bound material is eluted with a 0.1–0.5 *M* NaCl gradient (600 ml). Eluted fractions are analyzed by electrophoresis, and those containing collagen XIV are pooled, dialyzed against 50 m*M* Tris–HCl, 10 m*M* EDTA, 0.1 *M* NaCl, pH 8.0, then passed over a fast protein liquid chromatography (FPLC) MonoQ column equilibrated with the same buffer. Bound proteins are eluted with a 0.1–0.6 *M* NaCl gradient (30 ml). The peak eluting at ~0.4 *M* NaCl is concentrated and size fractionated by FPLC on Superose 6 (100 ml; Pharmacia LKB Biotechnology) equilibrated with 0.2 *M* ammonium hydrogen carbonate. The first eluted peak which contains collagen XIV, is pooled and stored at −20 °C.

Reduction and alkylation of collagen XIV (if necessary for further investigation) is carried out in 6 *M* guanidine–HCl, 0.1 *M*, 0.1 *M* Tris–HCl, pH 6.9, with 0.02 dithiothreitol (5 h, 37 °C). The reaction is stopped by adding a 4-fold molar excess of *N*-ethylmaleimide (2.5 h, 37 °C) followed by dialysis against 0.2 *M* ammonium hydrogen carbonate.

Alternatively also heparin–Sepharose column can be used for collagen type XIV preparation. However, in this procedure collagen type XIV coelutes with fibronectin from which it can be separated on a MonoQ column (where it is eluted at 0.25–0.30 *M* NaCl gradient) [96,97].

14. Type XV collagen

Type XV collagen was described for the first time by Myers et al. [98]. Its existence was revealed by isolating a human cDNA clone encoding a specific collagen sequence. No preparative procedure to our best knowledge has been reported so far. On the other hand data are available [99] indicating that collagen type XV (and types XIII, and XVIII as well) yield upon limited proteolysis bioactive fragments that are related to endostatins capable of inhibiting primary tumor growth. Preparation of the fragments can be done from blood ultrafiltrate and has been described in detail in the report of John et al. [99].

The fact that 10 000 l of human blood ultrafiltrate obtained from patients with chronic renal insufficiency is needed at the beginning represents a

serious limitation in preparation of these bioactive peptide fragments. For additional information see Section 17.

15. Type XVI collagen

This collagen type can be prepared on a micro-scale from a cell culture of fibroblasts (human skin) according to the following procedure: confluent cells are washed once or twice with phosphate-buffered saline (NaCl/P) and incubated in deficient Dubelcco's modified Eagle's medium (DMEM, without fetal bovine serum, sodium pyruvate, L-methionine and L-cystine) containing 50 μg/ml ascorbic acid at 37 °C under 5% CO_2 for 2–3 h. Cells are labeled in 0.8 ml fresh deficient DMEM containing 50 μg/ml ascorbic acid and 50 μCi L-[^{35}S]cysteine for 15–18 h. At the end of the labeling period, culture medium is collected and a 5-fold excess of the Immunomix buffer with protease inhibitors is added to give a final concentration of 10 m*M* NaCl/P, 1% Triton X-100, 0.5% sodium deoxycholate, 2 mg/ml bovine serum albumin (BSA), 5 m*M* iodine acetamide, 1 m*M* phenylmethanesulfonyl fluoride, 1 m*M* EDTA, 2 μg/ml leupeptin, 2 μg/ml pepstatin, and 2 μg/ml aprotinin. The medium is incubated on ice for 30 min before immunoprecipitation. Cells layers are fractionated further by differential extraction with SDS to generate a soluble fraction (referred to as cells) and insoluble fraction (referred to as the extracellular matrix, ECM), which is subsequently solubilized in 4 *M* urea. Both the cell and extracellular matrix fraction are subjected to a complex purification procedure and the resulting samples are loaded onto 4–15% SDS–polyacrylamide gradient gels and run at 40 mA/gel for 45 min. Gels are fixed in a solution of 45% methanol and 15% acetic acid, immersed in autoradiography enhancer solution (DuPont-NEN), dried and exposed to fluorography at −70 °C.

Immunoprecipitation with Col A 1 antibody of human dermal fibroblasts yields a major band of M_r 220 000 which is sensitive to bacterial collagenase and partially resistant to pepsin indicating the presence of collagenous and non-collagenous domains in the α_1 XVI molecule [100]. Preparation is possible from the polyacrylamide gel separations.

16. Type XVII

Collagen XVII, also known as the M_r 180 000 bullous pemphigoid antigen or BP180, is a structural component of the hemidesmosomes in epithelial cells [101]. The cDNA sequence predicts a type II integral transmembrane protein of 1497 amino acids, with an NH_2-terminal intracellular domain of 466 amino acids, a transmembrane domain of 23 residues and a C-terminal extracellular domain of 1008 amino acid residues [102,103]. Because of the 16 collagenous subdomains characterized by the –Gly–X–Y– repeat sequences, the molecule was designated collagen XVII [104,105].

Extraction of protein fraction containing collagen type XVII can be effected both from keratinocyte cultures or from epidermis.

From cell cultures the extraction is done by using a neutral buffer containing 1% Nonidet P-40, 0.1 *M* NaCl and 25 m*M* Tris–HCl, pH 7.4, and if necessary a set of proteinase inhibitors.

From epidermis the following procedure can be applied: the skin is subjected to epidermolysis in a neutral buffer containing 20 m*M* EDTA and proteinase inhibitors (overnight). The dermis and epidermis are mechanically separated and extracted with a buffer containing 8 m*M* urea and 0.05 *M* Tris–HCl, pH 6.8 (the buffer contains proteinase inhibitors). Extraction is done with 400 μl/cm^2 of the skin sample. Then, the extract is extensively dialyzed against 0.8 *M* urea, 2% SDS, and 5% glycerol in 0.1 *M* Tris, pH 6.8, and 5–15 μl are used for SDS–PAGE (7% polyacrylamide or 3–15% polyacrylamide gradient gels). The amount loaded on the gel should not be more than the mentioned 15 μl as otherwise the separation is obscured by the excess of co-extracted keratins.

Collagen type XVII α-chains and their sub-domains can be identified by immunoblotting; however, no strictly preparative procedures for this collagen type are available [103].

17. Type XVIII

A recently defined sub-family of non-fibrillar collagens, the multiplexins, is comprised of type XV and type XVIII collagens [106], and characterized by multiple interruptions in the central triple helical

domain and the presence of a unique non-triple-helical domain at the C-terminus (NC1) [107].

As demonstrated later the structure of the NC1 domain is nearly identical with endostatin (sometimes referred to as angiostatin) [108,109]. Originally endostatin was prepared from the hemangioendothelioma cell line and only later its structure was compared with the NC1 domain of type XVIII collagen. It was suggested that the presence of endostatin in the cell culture medium may reflect proteolytic release of NC1 in cell culture. The hemangioendothelioma endostatin is the shortest (its sequence starts at position 132 of the mouse NC1 domain which possesses 315 residues), human and mouse endostatins possess positions 129–312 and 130–315, respectively (the structure contains several endogenous cleavage sites and several amino acid interchanges and deletions between the mouse and human NC1 domain).

The separation of endostatin (angiostatin) from the cell culture medium is done by heparin–Sepharose chromatography during which it is eluted with 0.6–0.8 *M* NaCl,10 m*M* Tris, pH 7.4. The fraction obtained is further run through a Bio-Gel P-100 fine or Sephacryl S-200HR column. Chromatography on a C_4 phase (eluted with 40–45%, v/v, acetonitrile in water, containing 0.1% trifluoroacetic acid) serves final purification. The resulting product has a M_r~20 000 as revealed by SDS–PAGE.

In a representative purification 10 l of conditioned media containing 50 mg of protein are used to purify 2 μg of the inhibitor [108].

Recombinant NC1 domain can be, as expected, also purified by heparin–Sepharose affinity chromatography [109] and is eluted as two major bands of M_r 25 000 and 38 000 (as revealed by PAGE). Further separation on a Sepharose 12 column revealed two fractions of M_r~100 000 and 38 000. It was suggested that the M_r 25 000 protein is monomeric endostatin while the M_r~100 000 protein fraction is the trimeric form is trimeric form of the M_r 38 000 protein. It was also concluded that the trimeric form is non-covalently bound (Fig. 18) [109].

In the paper of Marneros and Olsen [107] it was suggested that N-terminal part of the NC1 domain bears the trimerization site; towards the C-end of the fragment follows a protease-sensitive hinge region

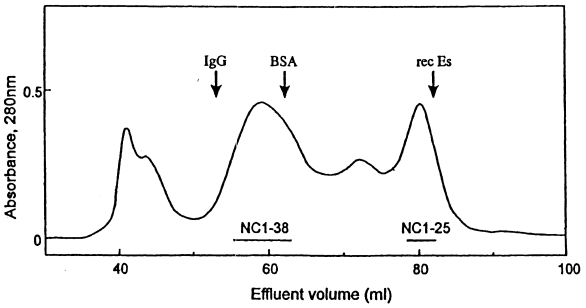

Fig. 18. Molecular sieve chromatography separation of oligomeric NC1 (NC1-38) from a monomeric fragment NC1-25. The Superose column is run in 0.05 *M* Tris–HCl, 0.5 *M* NaCl, pH 7.4, and calibrated with immunoglobulin G (IgG), bovine serum albumin (BSA) and recombinant mouse endostatin (rec Es). From Ref. [109] with permission.

and the very C-terminal M_r~20 000 domain corresponding to endostatin. The biological role of the NC1 domain and its fragments are far reaching; for further information see Ref. [107].

18. Types XIX–XXV collagens

To our best knowledge preparative procedures for these novel collagen types have not been published so far. The limited information about their structure (sequence and size of the parent polypeptide chains) has been derived from the sequence cDNA clones. Description of these procedures is clearly beyond the scope of the present review; however an idea about the general strategy used can be gained from a recent paper of Koch et al. [3] dealing with the identification of collagen α_1(XX) polypeptide chains. An idea about the available information regarding structure and occurrence of these collagen types is summarized in Table 3.

19. Miscellaneous

Already in 1983 Odermatt et al. [63] worked out a preparative procedure for the so-called intima collagen. It was revealed that this collagen type present in placenta can be released by pepsin treatment and is composed of two constituent α-chains of M_r 50 000 and 70 000; the chains are disulphide bonded

Table 3
A survey of fundamental characteristics of recently described collagens

Type	Chain size	Characteristics	Comment/Source	Refs.
XIX	1142 amino acids.	Belongs to FACIT family. Five subdomain triple helical region.	Present similarly to collagen type XV in vascular, neuronal, mesenchymal and some epithelial basement membrane zones. Related to angiogenic and pathological process.	[120,121]
XX	A polyclonal antibody against synthetic α_1(XX) polypeptide reacts with M_r 185 000, 170 000 and 135 000 units bands on polyacrylamide gel electrophoresis.	Belongs to FACIT family, similar to collagen types XII and XIV, but smaller than their short forms.	Present in corneal epithelium, sterna cartilage, tendon; not an abundant component. Binds to collagen fibrils through the C-terminal region with the N-end projected away from the fibril.	[3]
XXI	Unspecified.	Belongs to FACIT family. N-terminal signal sequence, followed by von Willebrand factor A-domain (one), thrombospondin domains (several) and an interrupted triple helix.	Present in heart, stomach, kidney, skeletal muscle and placenta.	[4,5]
XXII	Unspecified.	Unspecified.	Hair follicle.	[8]
XXIII	Unspecified.	Unspecified.	Cornea.	[6]
XXIV	Contains collagen types V and XI similar N-peptides (547 amino acids), a signal peptide (250 amino acids) N-terminal thrombospondin-like domain, a region with changed tyrosines and a minor triple helix.	Belongs to fibrillar collagens, close to collagens type V and XI.	Cartilage, retina, cornea, skin. It is likely to regulate the diameter of collagen fibrils.	[7]
XXV	Unspecified.	Contains three collagen-like Gly–X–Y repeat motifs, partially homologous to collagen type XIII.	Transmembrane protein involved in β-amyloidogenesis and neural degeneration.	[2]

and possess an unusually high thermal stability. Triple helical molecules are released by splitting the disulphide bonds and this reduced material can be cleaved by pepsin to yield a collagenous component containing α-chains of M_r 38 000. Treatment with bacterial collagenase releases two non-collagenous segments of M_r 15 000 and 30 000. Collagenase treatment before reduction yields a large fragment containing both collagenous and non-collagenous parts of the molecule in addition to a single non-collagenous segment. Basically this collagen is close to type VI, possesses a large glycosidic domain and can be classified as extracellular matrix glycoprotein.

The preparative procedure runs as follows: in order to isolate the parent α-chains and the collagenous and non-collagenous domains the purified material has to be subjected to proteolytic modification. Reduction and separation of chain constituents is another necessary step.

Digestion of intima collagen can be done for 24 h at an enzyme/substrate ratio of 1:100 (w/w). For digestion with pepsin at 20 °C intima collagen is dissolved in 0.5 *M* formic acid (2 mg/ml). For digestion with bacterial collagenase (to reveal the non-collagenous domain) the material is dialysed against 0.2 *M* NaCl, 2 m*M* $CaCl_2$, 50 m*M* Tris–HCl buffer, pH 7.4. Digestions are then done at 37 °C before or after heat denaturation. The extent of degradation can be evaluated by electrophoresis under reducing conditions and by molecular sieve chromatography on Sephadex G-100 column (100× 2.6 cm) equilibrated and eluted in 50 m*M* formic

acid. Collagenase digest (2 h, 37 °C) can be also prepared from partially reduced material and can be separated on a Sephacryl S-200 column (110×1.5 cm) equilibrated and eluted in 0.2 *M* ammonium bicarbonate buffer, pH 7.9. These procedures are only a small variation of methods applicable to collagen type VI.

For reduction under non-denaturating conditions samples are dissolved in 0.1 *M* acetic acid (2 mg/ml) and dialysed in at 4 °C against 0.4 *M* NaCl, 50 m*M* Tris–HCl buffer, pH 7.4. Reduction is then performed with various concentrations of dithiothreitol, 2-mercaptoethanol or cysteine at 15 °C for periods of 30 min to 24 h and stopped by addition of a 4-fold excess of ethyleneimine followed by 1 h incubation at 15 °C. Reagents are removed by dialysis against 20 m*M* sodium phosphate buffer, pH 7.2, and against water at 4 °C. The precipitated material is finally dissolved in 0.1 *M* acetic acid. For chromatography on CM-cellulose the material is dialysed at 4 °C against 1 *M* urea, 20 m*M* sodium acetate buffer, pH 4.8. Elution of the protein from the column is accomplished with a linear gradient of 0–0.4 *M* NaCl [110].

For the separation of the chain constituents the samples are completely reduced in 8 *M* urea and *S*-aminoethylated [111] in the next step. Purification steps include chromatography on an agarose Bio-Gel A-1.5m column (120×3 cm) equilibrated and eluted in 1 *M* $CaCl_2$–50 m*M* Tris–HCl buffer, pH 7.4, and on a CM-cellulose column as described above [63].

20. Micropreparative separations in polyacrylamide gels

Polyacrylamide gel electrophoresis represents a well applicable technique for micropreparation of collagen fragments in situations (quite frequent) when the collagenous material is scarce. These procedures are applied for (i) revealing fragments arising during collagen metabolism and (ii) revealing minor collagens and their constituting α-chains.

Before any micropreparation can be materialized it is necessary to detect the separated zones. For this purpose immunoblotting techniques are applied.

20.1. Recovery of collagenous material from polyacrylamide gels

Zones revealed by the staining procedure are cut out with a blade, the gel pieces are placed into a microcentrifuge tube, the dye is removed by adding 1 ml of wash buffer and sonicating for 5–15 min at 60 °C until clear gels. The wash buffer (methanol–acetic acid–water, 40:10:50, v/v/v) is removed, 50–100 μl of the extraction buffer (0.1 *M* $NaHCO_3$, 8 *M* urea, 3% SDS, 0.5% Triton X-150, 25 m*M* dithiothreitol) is added and incubated 20–30 min at 65 °C. The gel is then homogenized with an Eppendorf fitting pestle and the tube with homogenized gel is incubated at 50–60 °C overnight. Next 100 μl of the extraction buffer are added and the gel slurry is transferred into an Amicon Microcon inserted with Micropore inset. The tube is rinsed with 100 μl of extract buffer and this rinse is also transferred into the Micropore inset. Next the assembly is spun until all liquid is removed from the Micropore inset (13 000 *g*, 20–30 min). Collagen fragments are retained above the Microcon membrane; the sample is transferred to a new vial and lyophilized. For analyzing by capillary electrophoresis the lyophilysates are dissolved in 500 μl of the pH 2.5 or pH 4.5 background electrolyte (phosphate) buffer (the latter containing 0.1% SDS) [112].

20.2. Immunoblotting detection of collagenous proteins

The prove the collagenous nature of the fragments, after slab gel electrophoresis the proteins are transferred onto a nitrocellulose membrane in 15 m*M* sodium borate buffer, pH 9.2, 24 h, at 4 °C. Starting transfer power conditions are 25 V/250 mA, finishing conditions are 25 V/350 mA. Sheep anticollagen type I (or another type as necessary) antibodies are diluted 1:20 in 2% skimmed milk in phosphate-buffered saline (PBS) and the nitrocellulose membrane is incubated in this solution 1 h at room temperature, washed with 2% skimmed milk-PBS. The membrane is than incubated in horseradish peroxidase (HRP)-conjugated antisheep-goat antibodies solution diluted 1:500, 1 h at room temperature. After washing, the membrane is stained with HRP

substrate, 4-chloro-1-naphthol (15 mg in 5 ml of methanol), 10 m*M* Tris–HCl and 0.04% H_2O_2. The reaction is allowed to proceed in the dark for 15 min until all bands are visualized and the membrane is air dried for storage [113].

20.3. Separations of molecular mass standards

As a matter of fact calibration of polyacrylamide gels in terms of molecular size is routinely done by a standard set of proteins. However as revealed from polyacrylamide gel electrophoresis of collagen type I parent chains and their polymers, these proteins exhibit an irregular behaviour, which results in about 20% increase in M_r estimate if calibration is done with non-collagenous proteins. Moreover, as polyacrylamide gel electrophoresis of at least abundant collagen types is not governed by molecular sieving only and represents a multimodal separation process, it is possible to separate molecular entities of equal molecular size (typically α_1 and α_2 parent chains of collagen type I). The physico-chemical basis of this effect (through it is known for years) is not known. It is neither a charge nor a structural effect as the separated chains possess minimum charge difference and the separation can be observed with native as well as denaturated collagen chains (and to a lesser extent with their polymers as well) [114]. It is likely that the difference in behaviour is related to the mesh-structure of polyacrylamide gels and as if the concentration of polyacrylamide in the gel is decreased (e.g., using polyacrylamide–agarose gels instead of pure polyacrylamide) this effect is abolished and typically the two parent α-chains of type I collagen comigrate and form a single zone only. This fact plays apparently a considerably less distinct role with collagenous proteins possessing non-collagenous domains. To our best knowledge there is not any report available from the literature concerning this problem.

Isolation of marker peptides arising after CNBr cleavage of collagen type I is based on a set of chromatographic procedures involving ion-exchange chromatography, reversed-phase chromatography and size-exclusion separations [114]. The general strategy employs a combination of ion-exchange chromatography and reversed-phase separation. Purity of the fractions obtained should be checked by PAGE and, if sufficient material is available, also by amino acid analysis.

CM-cellulose chromatography, generally following the procedure of Butler et al. [115] is used. A column (15×1.5 cm) is packed with CM-cellulose and operated at 42 °C. The column is equilibrated with 0.02 *M* citrate buffer, pH 3.6, and eluted at 100 ml/h with a linear ionic strength gradient from 0.02 to 0.14 *M* (2000 ml in total). The amount of peptides loaded is about 100 mg, the effluent is monitored at 220 nm and the corresponding fractions from five runs are pooled and lyophilized.

Size-exclusion chromatography follows as the next step: two columns (30×0.75 cm) in tandem are used (Bio-Sil, SEC-125, Bio-Rad); elution is isocratic with 0.5 *M* guanidine–HCl, 50 m*M* Tris, pH 7.5, at a flow-rate of 0.6 ml/min. The effluent is monitored at 220 nm (the method was described by Miller et al. [116]). With larger peptides (above M_r 25 000), the gel permeation step can be performed with an Ultrogel AcA-54 column (40×1.6 cm) at 20 °C and elution at 40 ml/h with 0.02 *M* citrate buffer, pH 3.6. The effluent is monitored at 230 nm and recycled three times [114].

A Vydac TP 201 column chromatography (25× 0.46 cm, 30 nm pore size) is used with a linear gradient of 12.8–44.8% acetonitrile in water at a flow-rate 1 ml/min over 1 h (with 0.01 *M* heptafluorobutyric acid used as the ion-pairing agent) [117] and can be used to compare the molecular masses of peptides separated in the two previously described chromatographic steps. Polyacrylamide gel electrophoresis (12% gel) is routinely used for checking the purity of isolated peptides [114].

21. Identification and purity assessment of the products

The result of all preparative procedures is a precipitate or lyophilized material that always needs at least some purity confirmation.

Current methods are based either on the application of additional separation techniques or on immunochemical detection. The latter approach can be (however, not necessarily) combined with poly-

acrylamide gel electrophoresis; for micropreparative procedures this is nearly a must, for localization of collagens (collagen types), e.g., in tissues. No preparative procedures are involved. As the typical collagenous core is a weak antigen, immunochemical reactions are preferably directed towards globular domains of the molecule (which are available particularly with the minor collagen types, see the FACIT subfamily).

Regarding identification procedures based on further separation, polyacrylamide gel electrophoresis is widely applied. This approach not only gives an idea about the relative molecular mass of the isolated proteins, but allows also to introduce depolymerization reactions which reveal information about chain composition (typically disulphide bond cleavage in collagens possessing this type of cross-linking or on the distribution of non-collagenous domains). As far as the structure of parent chains is concerned, enzymatic treatment offers the most direct approach for obtaining this information. If the collagenous structure contains globular (non-collagenous) domains, they can be split by a number of non-specific proteases by which this part of the molecule is cleaved into smaller fragments (low-molecular-mass peptides) leaving the collagenous core intact. The other approach is based on just the opposite philosophy. The product is treated by bacterial collagenase by which procedure the collagenous part of the molecule is split into (mostly) tripeptides leaving the globular (non-collagenous) domains intact.

Finally, instead of enzymatic cleavage it is possible to apply purely chemical degradation by cyanogen bromide. However, this approach is limited to collagenous structures which possess low proportion of methionine residues and, consequently, yield a limited number of fragments. Typically with collagen types I, III and V CNBr marker peptides can be separated by capillary electrophoresis, and because the result of this separation is a peak, not a zone, these peptides can be quantitated by peak area and the proportion of, e.g., co-precipitated collagens, can be revealed. However, this approach has three hidden dangers: first all collagens owing to the Gly–Pro–X sequence tend to stick to the inner wall of the capillary more than all other proteins. Second owing to the large internal homogeneity of at least the triple helical domains in all collagens, the physico-chemical properties of the related peptides are quite close, which does not offer favourable prerequisites for successful separations. Third, owing to the uneven distribution of methionine in filaments forming collagens (other collagen types have not been subjected to our best knowledge to this treatment so far), the resulting peptides span over a wide range of M_r (1000–66 000). Because the amount of the sample is nearly always scarce, the separation is difficult to divide into more steps that would result in more simple peptide mixtures [112].

Admittedly, polyacrylamide gel electrophoresis offers more clear cut results; however, it offers semiquantitative information at best and the very small peptides that have a poor (if any) affinity to the staining dye cannot be visualized.

Finally it has to be emphasized that fibre forming collagens owing to their low metabolic turnover are ideal candidates for non-enzymatic posttranslational modifications (typically glycation, additional cross-link formation and deposition of age pigments). From the strictly chemical point of view all these represent separate chemical entities. To separate these (both on the analytical and preparative scale) is practically impossible at the level of current separation technology as the changes over the molecule are always only a few and more than one type of modification reaction can occur side-by-side and so do not result in a distinct change in physico-chemical properties preventing thus the modified entities from both chromatographic and electromigration separation. This problem can be overcome by a deep collagen fragmentation and comparison of the resulting low molecular mass peptide maps. As straightforward as this approach may seem to be, its materialization is not easy. This can be illustrated best with collagen type I which, according to its known primary structure yields 220 peptides (96 peptide species) after bacterial collagenase cleavage.

Applying capillary electrophoresis at extremely low pH values (2–2.5) offers acceptable peptide maps; however, quantitation of individual peptide peaks is not possible: the overlaps are much too heavy and only a very limited number of standards is commercially available (most of the peptides would have to be custom synthesized). The only reasonable way how to evaluate these maps is valley-to-valley

area estimation (i.e., determination an area covered by a cluster of well separated peaks) followed by comparison of the data obtained by principal component analysis [118].

The better way to obtain a reasonable separation of a complex peptide mixture (in this case the separated peptide set originated from a mixture of collagen types I and III) is to combine reversed-phase chromatography and capillary electrophoresis [119]. In this procedure the mixture of peptides arising after collagen digestion with bacterial collagenase is separated by HPLC on a C_{18} column (in a water–acetonitrile gradient with trifluoroacetic acid); five fractions of peptides are collected and further separated by capillary electrophoresis at 50 m*M* phosphate buffer, pH 2.5. In this way it is possible to separate 154 peptide peaks (in contrast to the 65 peaks revealed during capillary electrophoresis only).

22. Conclusions

At the moment procedures for the fiber forming collagens are well established (a number of them is 20 and even more years old). With the more recently discovered members of the collagen superfamily the preparation procedures tend to be micropreparative; for a number of them adequate preparative procedures even do not exist and their presence in tissues is based on immunofluorescence techniques exploiting antigens directed to known immunologically responsive sequence derived from DNA databases. As these minor collagens represented (by definition) by a set of proteins possessing both typically collagenous (sometimes very short) and non-collagenous domains, are present in tissues at extremely low concentrations, the preparative procedures are or could be based on micropreparative approaches using polyacrylamide gel separations which follows a simple extraction procedure.

Surprisingly enough, immunoaffinity chromatographic (or electromigration) techniques that are likely to be very perspective in this direction have not been published to our best knowledge so far.

Identification and purity assessment is based on immunochemical reactions or on the evaluation of complex peptide maps resulting from either CNBr or collagenase cleavage. For the determination of non-collagenous and collagenous domains non-specific protease and bacterial collagenase cleavage procedures are widely applied.

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