Embryonic chick cartilage collagens

Differences in the low-M_r species present in sternal cartilage and tibiotarsal articular cartilage

C.M. Kielty, D.J.S. Hulmes*, S.L. Schor+ and M.E. Grant†

*Departments of Biochemistry and *Medical Biophysics, University of Manchester Medical School, Oxford Road, Manchester M13 9PT,* and *+Cancer Research Campaign Department of Medical Oncology, Christie Hospital and Holt Radium Institute, Manchester M20 9BX, England*

Received 19 January 1984; revised version received 22 February 1984

The collagenous polypeptides present in embryonic chick sternal and tibiotarsal cartilages have been solubilised by digestion with pepsin and separated by salt fractionation. Type II collagen, $1\alpha 2\alpha 3\alpha$ collagen, and two polypeptides (apparent molecular mass 150 and 42 kDa), which were reducible to a number of smaller peptides, were extracted from both tissues. However, also present in the peptic digests of tibiotarsal cartilages was a major non-reducible highly-soluble polypeptide of 45 kDa. This short-chain collagen is apparently identical to the pepsinized product of G collagen (M_r 59000), a major low-M_r procollagen-like species previously detected in chick chondrocyte cultures.

Cartilage *Sternum* *Tibiotarsus* *Short-chain collagen*

1. INTRODUCTION

Recent studies on the collagenous components of cartilagenous tissues have demonstrated the presence of a number of minor low-M_r collagens in pepsin digests of neonatal pig and human cartilage [1–3], bovine nasal cartilage and human intervertebral disc [4,5], foetal calf cartilage [6], and chick sternal cartilage [7–9]. The nomenclature and molecular sizes assigned to the various fragments have caused considerable confusion in the literature. However, there is general agreement that cartilages contain collagen chains which are highly disulphide-bonded, and which can be recovered after reduction of the pepsin-resistant fraction as very soluble low-M_r peptides. These fragments must derive from larger collagenous proteins which may have several pepsin-labile sites in the triple-helical domains, but whether they derive from one or more precursor molecules is not known.

Investigation of collagen synthesis by embryonic chick sternal chondrocytes in culture has provided evidence for the existence of two low-M_r disulphide-bonded collagens, designated H and J collagen chains (M_r 69000 and 84000, respectively), which may represent the parent molecules of some of the pepsin-extracted species isolated from cartilagenous tissues [10]. In these biosynthetic studies considerable interest has also focussed on another small procollagen-like species (designated G collagen [10–12]) which comprises polypeptides of 59 kDa, does not contain inter-chain disulphide links, and contains a pepsin-resistant helical domain (designated Gp) of 45 kDa. The G collagen is the major collagen synthesised by sternal chondrocytes maintained in long-term culture in collagen gels and an apparently identical short-chain collagen species is secreted by aged endochondral chondrocytes from chick embryos [13–17]. Evi-

† To whom reprint requests should be addressed

Published by Elsevier Science Publishers B.V.
00145793/84/$3.00

dence for the existence of G collagen in intact chick cartilages has not been established but here we describe the occurrence of a collagenous polypeptide of 45 kDa, analogous to Gp, in extracts of tribiotarsal cartilage of 17-day-old chick embryos.

2. MATERIALS AND METHODS

2.1. Preparation and purification of collagens

Sterna and tibiotarsi (zones 1–3 inclusive [18,19]) were dissected from 17-day-old chick embryos and all adhering tissues removed. The cartilages from the distal end of the tibiotarsi were dissected out, frozen in liquid N_2 and crushed to a powder. The preparation was digested by pepsin (E:S of 1:100) in 0.1 M acetic acid for 24 h at 4°C. The digests were clarified by centrifugation at $10000 \times g$ for 30 min. The collagenous components were fractionated at 0.8, 1.2 and 2.0 M NaCl under acidic conditions as in [7], then redissolved in and extensively dialysed against 0.1 M acetic acid, before lyophilisation. Low-M_r collagens precipitated at 2.0 M NaCl were further purified by CM–cellulose chromatography.

2.2. Characterisation of collagens

Discontinuous SDS-polycarylamide slab gel electrophoresis (SDS–PAGE) was carried out on the fractionated collagens with and without prior reduction by 5% (w/v) β-mercaptoethanol [10]. Proteins were visualised by Coomassie brilliant blue staining [20] and their M_r values determined using α1(I) and α1(II)chains and CNBr digestion peptides derived therefrom as standards. The susceptibilities of the isolated polypeptides to digestion by highly purified bacterial collagenase (Advanced Biofactures, Lynbrook, NY) were ascertained using the incubation conditions in [10].

Samples for amino acid analysis were hydolysed in 6 M HCl at 110°C for 18 h under vacuum, then analysed using a JEOL-6AH amino acid analyser.

Segment long spacing crystallites were prepared by dialysis of unreduced purified native collagen fractions against 0.2% (w/v) ATP in 0.1 M acetic acid at 4°C for 2 days. Samples were applied to thin carbon films and negatively stained with 1% phosphotungstic acid. Grids were examined in a JEOL 100B electron microscope.

3. RESULTS AND DISCUSSION

Pepsin-solubilised peptides isolated from 17-day-old chick embryo sternal and tibiotarsal cartilages were separated by salt fractionation in acid conditions and then analysed by SDS–PAGE. The majority of polypeptides (approx. 84% of the solubilised polypeptides) precipitated at 0.8 M NaCl and, as expected, this fraction contained type II collagen chains, although the occurrence of a minor band migrating in the position of the α2(I)collagen chains in the tibiotarsal cartilage sample indicated that a small amount of type I collagen was also present (not shown).

The fractions precipitating at 1.2 and 2.0 M NaCl were analysed under non-reducing and reducing conditions (fig.1). Significant differences were noted in the polypeptide compositions of the fractions from the different cartilages. It should be noted that all the polypeptides in fig.1 were susceptible to digestion by highly purified bacterial collagenase except those that corresponded to the polypeptides of pepsin used to solubilise the collagens (see fig.1). The 1.2 M NaCl fraction from sternal cartilage consistently contained only high-M_r polypeptides when analysed under non-reducing conditions (fig.1, track 5): 3 bands of equal intensity were observed migrating in the positions expected for $1\alpha 2\alpha 3\alpha$ collagens [21] and their mobilities were unaffected by reduction (fig.1, track 1). Electrophoretic analyses of the 2.0 M NaCl sternal fraction revealed the presence in the unreduced sample of a diffuse band of α-chain size and major bands of approx. 150 and 42 kDa (fig.1, track 7). The latter two components are analogous to the chick sternal collagenous polypeptides described variously as HMW and LMW [7,8] and M1 and M2 [9]; and the diffuse band of approx. 110 kDa is likely to represent a breakdown product of the large species [9]. Reduction of this sternal fraction yielded a number of peptides of lower molecular mass whose sizes were calculated to be approx. 100, 55, 48, 36, 31 and <20 kDa (fig.1, track 3). Polypeptides of similar sizes and properties were identified by authors in [7,9] in studies on chick sternal cartilage.

The 1.2 M NaCl fraction from tibiotarsal cartilage contained bands migrating in the positions of $1\alpha 2\alpha 3\alpha$ chains as well as the disulphide-bonded polypeptides similar to those seen in the 2.0 M

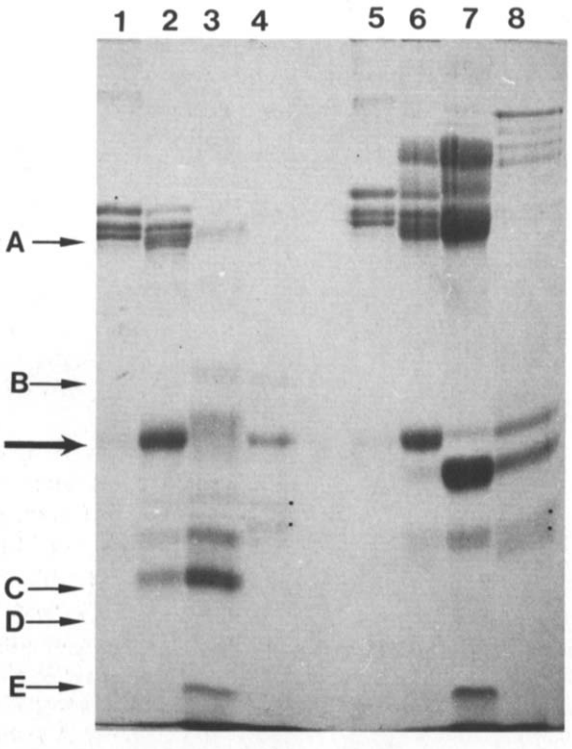


Fig.1. SDS–PAGE (8%) of the 1.2 and 2.0 M NaCl precipitates from peptic digests of embroynic chick sternal and tibiotarsal cartilages. Samples were 1.2 M NaCl precipitates from sternal cartilage (1,5) and tibiotarsal cartilage (2,6), and 2.0 M NaCl precipitates from sternal cartilage (3,7) and tibiotarsal cartilage (4,8). Samples in tracks 1–4 were run after reduction with β-mercaptoethanol whereas tracks 5–8 contain unreduced samples. The migration of collagenous standards of known M_r is indicated: (A) α1(II)chains (95 kDa), (B) α2(I)CB3,5 (61 kDa), (C) α2(I)CB4 (29.5 kDa), (D) α1(I)CB8 (25.1 kDa), (E) α1(I)CB6 (20 kDa). The major non-disulphide-linked short-chain collagen (45 kDa) of interest is also arrowed. Bands corresponding to pepsin are indicated by small dots on the electrophoretogram.

NaCl sternal fraction, when analysed under non-reducing and reducing conditions. In addition, a major component of 45 kDa which was unaffected by reduction, was also present (fig.1, tracks 2 and 7). This non-reducible 45 kDa collagenous polypeptide was also present in the 2.0 M NaCl tibiotarsal fraction (fig.1, tracks 4 and 8) and was a major band present when the latter fraction was analysed under reducing conditions.

This collagenous polypeptide (45 kDa) has not been described in tissue extracts, but a non-reducible component of identical electrophoretic mobility (designated Gp) has been obtained as the pepsinised product of G collagen synthesised by embryonic chick sternal chondrocytes in culture [10–12]. A similar, if not identical, molecule to G collagen has also been described in cultures of chondrocytes derived from chick tibia [13–17] and in organ cultures of rabbit growth plate cartilage [22]. Purification of the 45 kDa component from the 2 M NaCl precipitate of the tibiotarsal cartilage extract was achieved by CM–cellulose chromatography under non-denaturing conditions (fig.2). Two peaks were eluted by the NaCl gradient: the first (peak 1) which exhibited high absorbance contained only small amounts of disulphide-linked collagenous peptides which migrated on reduction with approximate molecular masses of 31 and 36 kDa, whereas the second more basic peak (peak 2) contained only the species of 45 kDa (fig.2b). Pepsin was unretained and appeared in the pre-gradient volume.

Table 1

Amino acid composition of non-disulphide-linked short-chain collagen (45 kDa) and Gp collagen isolated from chondrocyte cultures

	Residues/1000 amino acids	
	Short-chain collagen (45 kDa)	Gp collagen
4-Hyp	101	107
Asp	41	37
Thr	18	15
Ser	30	22
Glu	93	90
Pro	113	117
Gly	312	312
Ala	65	60
Cys	–	–
Val	20	22
Met	14	15
Ile	22	25
Leu	56	56
Tyr	2	2
Phe	13	11
Hyl	31	32
Lys	26	25
His	7	7
Arg	37	34

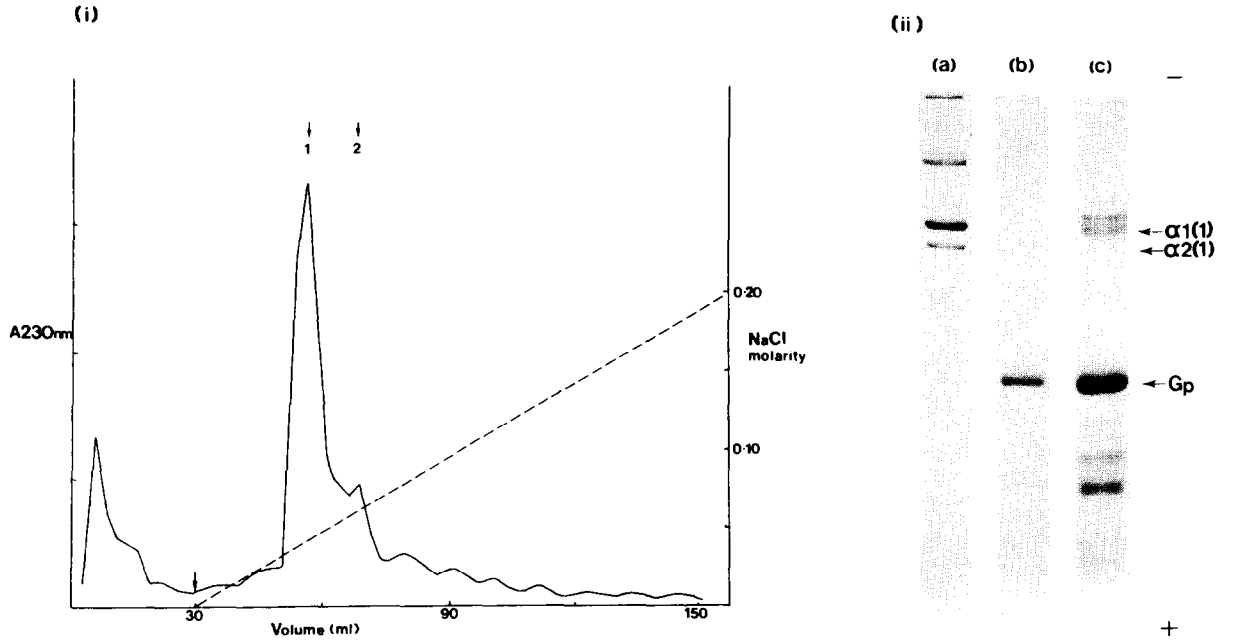


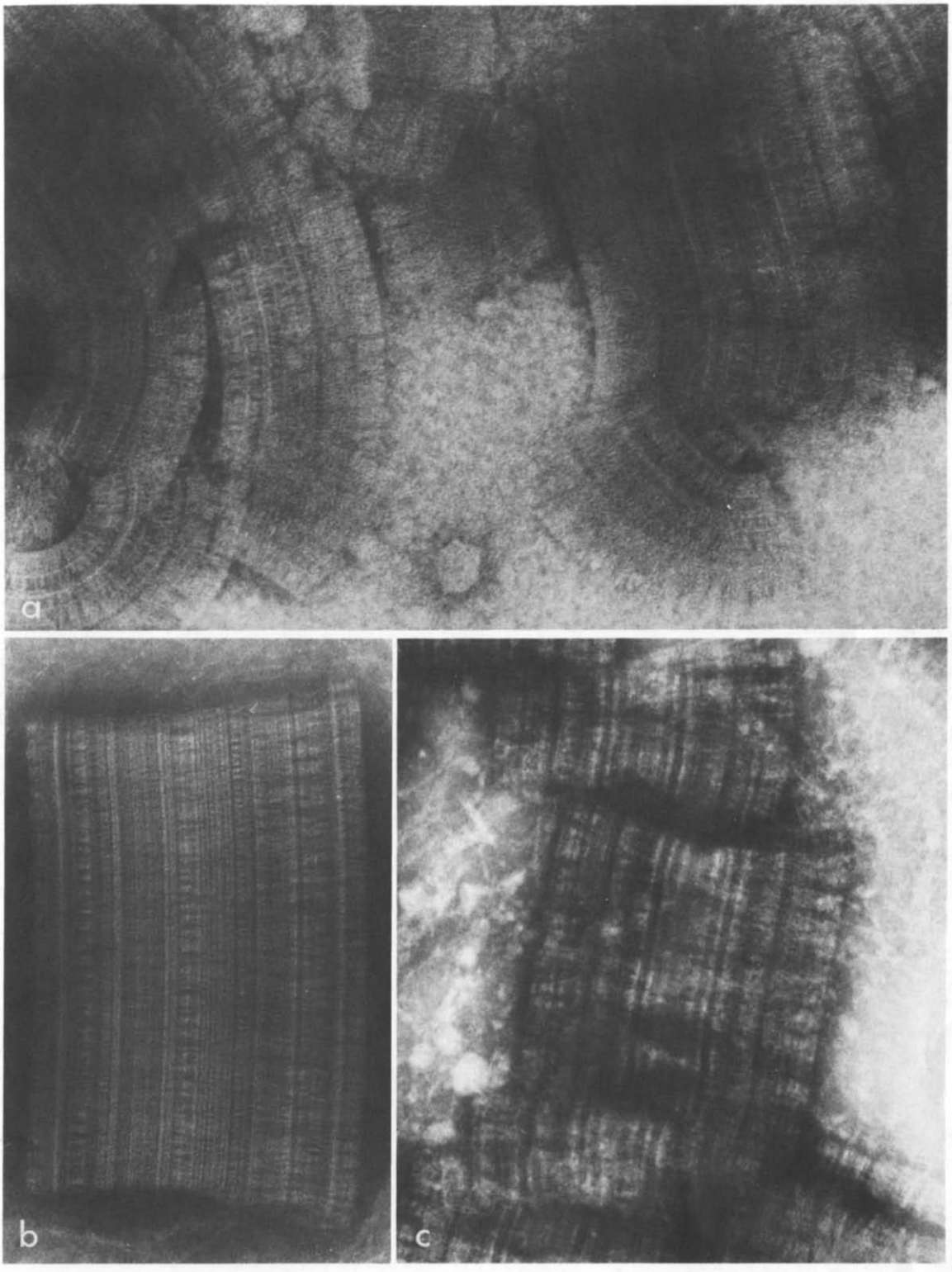
Fig.2. Purification of 45 kDa collagenous species from peptic digest of tibiotarsal cartilage. (i) The fraction precipitated at 1.2 M NaCl was applied to a CM–cellulose column eluted with an NaCl (0–0.2 M) gradient. (ii) Samples recovered from the column were analysed by SDS–PAGE: track a, standard type 1 collagen; track b, material eluted in peak 2; track c, sample applied to column.

Amino acid analysis of the purified 45 kDa component was virtually identical to that of the Gp chain recovered from 17-day-old chick embryo tibiotarsal chondrocytes cultured as in [15] and purified by ion-exchange chromatography as above (table 1). Both collagenous peptides lack cysteine but have relatively high methionine, aromatic amino acid and hydroxylysine contents when compared to the well characterised interstitial collagens. The methionine content is consistent with our previous observation [12] that CNBr digestion of G collagen produces only peptides of very low M_r; and the hydrophobic nature of the protein may contribute to the stability of this short-chain triple helical collagen.

The purified short-chain collagen (45 kDa) recovered off the CM–cellulose column was used to prepare segment-long-spacing (SLS) crystallites. The banding pattern obtained was distinct from chick type I and II collagens. The SLS crystallites of this short-chain collagen were approx. 130 nm in length corresponding to about 43% of the normal α-chain size, and they tended to form dimeric aggregates as shown in fig.3.

All previous data on G collagen have derived from cell culture [10–15] or organ culture [22] experiments. The presence of detectable levels of the pepsinised product of G collagen in chick embryo tibiotarsal cartilage described here provides corroborative evidence that this polypeptide is synthesised particularly by hypertrophying chondrocytes in zone 3 of this epiphyseal growth region ([13–15], and unpublished). No evidence was obtained for the presence of G collagen polypeptides in the sternal cartilage but previous studies demonstrated that chondrocytes from this tissue can be induced to synthesise G collagen when cultured in serum depleted of fibronectin [10–12]. Further experiments are currently underway to determine the function of this unusually stable low-M_r collagen,

Fig.3. Electron micrographs of SLS crystallites. (a) SLS monomers (left) and symmetrical dimers (right) of short-chain collagen (45 kDa). (b) Chick type II collagen. (c) SLS dimers of short-chain collagen (45 kDa). All samples negatively stained with 1% PTA, pH 7. ×200 000.


a
b
c

to ascertain its precise localisation within cartilagenous tissues, and to elucidate the control exerted by the extracellular matrix on the synthesis of G collagen.

ACKNOWLEDGEMENTS

We are grateful to Dr Adrian Shuttleworth and Mrs Joan Ward for the amino acid analyses. This work was supported by grants from the Medical Research Council. D.J.S.H. is an MRC senior fellow.

REFERENCES

[1] Shimokomaki, M., Duance, V.C. and Bailey, A.J. (1980) FEBS Lett. 121, 51–54.
[2] Shimokomaki, M., Duance, V.C. and Bailey, A.J. (1981) Biosci. Rep. 1, 561–570.
[3] Duance, V.C., Shimokomaki, M. and Bailey, A.J. (1982) Biosci. Rep. 2, 223–227.
[4] Ayad, S., Abedin, M.Z., Grundy, S.M. and Weiss, J.B. (1981) FEBS Lett. 123, 195–199.
[5] Ayad, S., Abedin, M.Z., Weiss, J.B. and Grundy, S.M. (1982) FEBS Lett. 139, 300–304.
[6] Ricard-Blum, S., Hartmann, D.J., Herbage, D., Payen-Meyran, C. and Ville, G. (1982) FEBS Lett. 146, 343–347.
[7] Reese, C.A. and Mayne, R. (1981) Biochemistry 20, 5443–5448.
[8] Reese, C.A., Wiedemann, H., Kuhn, K. and Mayne, R. (1982) Biochemistry 21, 826–829.
[9] Von der Mark, K., Van Menxel, M. and Wiedemann, H. (1982) Eur. J. Biochem. 124, 57–62.
[10] Gibson, G.J., Kielty, C.M., Garner, C., Schor, S.L. and Grant, M.E. (1983) Biochem. J. 211, 417–426.
[11] Gibson, G.J., Schor, S.L. and Grant, M.E. (1981) Biochem. Soc. Trans. 9, 550–551.
[12] Gibson, G.J., Schor, S.L. and Grant, M.E. (1982) J. Cell Biol. 93, 767–774.
[13] Schmid, T.M. and Conrad, H.E. (1982) J. Biol. Chem. 257, 12444–12450.
[14] Schmid, T.M. and Conrad, H.E. (1982) J. Biol. Chem. 257, 12451–12457.
[15] Schmid, T.M. and Linsenmeyer, T.F. (1983) J. Biol. Chem. 258, 9504–9509.
[16] Capasso, O., Gionti, E., Pontarelli, G., Ambesi-Impiombato, F.S., Nitsch, L., Tajana, G. and Cancedda, R. (1982) Exp. Cell Res. 142, 197–206.
[17] Mayne, R., Reese, C.A., Williams, C.C. and Mayne, P.M. (1983) in: Limb Development and Regeneration, part B, pp. 125–135, Alan R. Liss, New York.
[18] Oohira, A., Kimata, K., Suzuki, S., Takata, K., Suzuki, I. and Hoshino, M. (1974) J. Biol. Chem. 249, 1637–1645.
[19] Kim, J.J. and Conrad, H.E. (1980) J. Biol. Chem. 255, 1586–1597.
[20] Fairbands, G., Steck, T.L. and Wallach, D.F.H. (1971) Biochemistry 10, 2606–2617.
[21] Burgeson, R.E. and Hollister, D.W. (1979) Biochem. Biophys. Res. Commun. 87, 1124–1131.
[22] Remington, M.C., Bashey, R.I., Brighton, C.T. and Jimenez, S.A. (1983) Collagen Rel. Res. 3, 271–278.