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High-performance liquid chromatographic quantification of allysine as bis-*p*-cresol derivative in elastin

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Summary. The first step in normal cross-linking in elastin is the formation of α-aminoadipic-δ-semialdehyde, allysine, through oxidative deamination of specific peptidyl lysine by the enzyme lysyl oxidase (EC 1.4.3.13). For the analysis of allysine, allysine was derivatized with *p*-cresol. The derivatization was carried out by acid hydrolysis (6N HCl containing 5% (w/v) *p*-cresol at 110°C for 48 h) accompanied with the hydrolysis of elastin. A bis-*p*-cresol derivative of allysine was isolated from bovine ligamentum nuchae elastin hydrolysates, and was characterized by UV, FAB-MS and NMR. This derivative was identified as 2-amino-6,6-bis(2-hydroxy-5-methylphenyl)hexanoic acid. A rapid, sensitive reverse-phase high-performance liquid chromatographic method with UV detection was developed for the quantitative determination of allysine as its bis-*p*-cresol derivative. The lower limit of detection of the bis-*p*-cresol derivative was 58 pmol in the standard sample with a 20-μl injection at a signal-to-noise ratio of 3. This method was applied to the determination of allysine in bovine ligamentum nuchae, aorta, lung, and rat aorta elastin. The allysine content in rat aorta elastin dramatically increased from 1 week to 2 weeks of age.

Keywords: Amino acids – Allysine – Cross-link – Desmosine – Elastin – Isodesmosine

Abbreviations: ACPP, 1-amino-1-carboxy-5,5-bis-*p*-hydroxyphenylpentane; APC, allysine-bis-*p*-cresol derivative; EDTA, ethylenediaminetetraacetic acid; HPLC, high performance liquid chromatography; P_2O_5, *di*-phosphrous pentaoxide; FAB-MS, fast atom bombardment-mass spectrometer; UV, ultra violet; NMR, nuclear magnetic resonance; HSQC, heteronuclear single quantum coherence

Introduction

Elastin is a core protein in elastic fibers and a rubber-like protein, which possesses unique elasticity and tensile strength. Elastin gives a resilient

elasticity to many tissues functioning physiologically. In general, it is thought that flexible, randomly coiled polymer chains, joined together by cross-links into an extensible, three-dimensional network characterize their elastomers (Rosenbloom et al., 1993).

The elastin cross-links develop after enzymatic conversion of specific peptidyl-lysyl residues to α-aminoadipic-δ-semialdehyde, allysine (Pinnell and Martin, 1968). Allysine undergoes the aldol condensation reaction with a neighboring allysine to form allysine-aldol (Lent et al., 1969). Then allysine-aldol interacts with an ε-amino group of peptidyl-lysyl residue to form merodesmosine (Starcher et al., 1967). A peptidyl-allysine also interacts with an ε-amino group of a neighboring peptidyl-lysyl residue to form lysino-norleucine (Franzblau et al., 1965; Lent and Franzblau, 1967). Three peptidyl-allysines fuse with a peptidyl-lysyl residue to form pyridinium cross-links, such as desmosine and isodesmosine (Partridge et al., 1963; Thomas et al., 1963; Anwar and Oda, 1966). Desmosine and isodesmosine are found exclusively in elastin. They greatly contribute to the maintenance of three-dimensional structure, the resilient elasticity, and strength of elastin. Allysine residues in elastin, therefore, are inevitable and crucial compounds for the formation of its cross-linked structure, which play an important role in connective tissue turnover; the precise quantification of allysine may be a key for elucidating several physiological phenomena associated with functional changes of elastin.

Several methods have been reported for the assay of desmosines in biological fluids and tissues (Yamaguchi et al., 1987; Salomoni et al., 1991; Soskel, 1987; King et al., 1980). Conventional amino acid analysis could not detect underivatized allysine; the allysine has been previously identified and determined by derivatizing it to chloronorleucine by reduction, α-aminoadipic acid by oxidation, and to α,ε-diaminopimelic acid and ε-hydroxy-α-aminopimelic acid by reaction with CN^- and NH_3 (Lent et al., 1969; Pereya et al., 1974; Miller et al., 1967; Paz et al., 1976). These derivatization methods, however, are complex and time-consuming.

Recently, we prepared a bis-phenol derivative of allysine, 1-amino-1-carboxy-5, 5-bis-*p*-hydroxyphenylpentane (ACPP) and developed its analytical method using phenol (Jahanmard and Suyama, 2000). In this paper, we have modified this previous method, and described the derivatization of allysine with *p*-cresol by acid hydrolysis and the HPLC method for quantitative analysis of the bis-*p*-cresol derivative of allysine.

Material and methods

Reagents and chemicals

Acetonitrile (HPLC grade), *p*-cresol, sodium dihydrogen phosphate, sodium borohydride ($NaBH_4$), hydrochloric acid, ethylenediaminetetraacetic acid (EDTA) (all analytical grade), *di*-phosphrous pentaoxide(P_2O_5) and dimethyl sulfoxide ($DMSO_d$, for NMR spectroscopy) were purchased from Nakalai Tesque (Kyoto, Japan).

Spectroscopy apparatus and chromatographic conditions

Positive ion fast atom bombardment (FAB)-mass spectra were obtained using a JEOL JMS700 mass spectrometer operated at an accelerating voltage of +10 kV. The FAB gun was operated at an accelerating voltage of 6 keV with an emission current of 10 mA and using xenon atoms as the bombarding gas. APC was mixed with glycerol/methanol. The UV spectra for APC was obtained with a UV-2100S spectrophotometer (Shimadzu, Kyoto, Japan), samples being dissolved in methanol. All NMR measurements were performed at 25°C using a Varian Unity 500 NMR spectrometer operated at 500 MHz and 125 MHz for ^{1}H and ^{13}C NMR experiments, respectively. Chemical shifts of ^{1}H and ^{13}C were determined with tetramethylsilane at 0.0 ppm as internal reference. ^{1}H resonance assignments were obtained using ^{1}H-^{1}H correlated spectroscopy (^{1}H-^{1}H COSY). The ^{13}C resonance from carbons with a directly attached proton were assigned by heteronulcear single quantum coherence (HSQC) spectroscopy. HSQC spectroscopy was the pulse field gradient standard Varian program GHSQC. All NMR spectra were processed with the Varian VNMR software package. Structural assignments of APC were made from sample solutions in $DMSO_d$.

The HPLC system consisted of a SHIMADZU (Kyoto, Japan) LC-6A pump, a SPD-6AV UV-VIS spectrophotometric detector, a C-R5A data station, and a HITACHI (Tokyo, Japan) L-7300 column oven. Separation was performed with a LiChrosper 100 RP-18 125-4 reverse-phase column (Merck, Darmstadt, Germany). A guard column (LiChrosper 100 RP-18, 4 × 4 mm I.D., 5 μm particle size; Merck, Darmstadt, Germany) was placed just before the inlet of the analytical column to reduce contamination of the analytical column. The mobile phase was composed of 0.05M sodium dihydrogen phosphate (pH2.2)-acetonitrile (3:1). The flow-rate was 1.0 ml/min and the column temperature was 30°C. The UV signal was monitored at 282 nm, which corresponds to an absorption maximum for APC.

Preparation of the elastin

The elastin from various bovine tissues and rat aorta were purified by the modified technique with 1M NaCl treatment described previously (Nakamura and Suyama, 1994) as follows. After removing peripheral connective tissue and lipids, bovine tissues (wet weight 10 g) cut into small segments with a mixer were suspended in 1M NaCl (50 ml) for 24 hr. The supernatant was discarded after centrifugation, and then the pellet was resuspended in 1M NaCl. This extraction process was repeated three times. The insoluble residue was washed with distilled water. After centrifugation, the insoluble residue was washed with excess of distilled water and defatted with chloroform/methanol (2:1, v/v) for 48 hr, being washed with ethanol and ether. The prepared elastin was dried *in vacuo* under P_2O_5. The elastin, which was purified by procedure A, contained polymeric collagen. Bovine ligamentum nuchae elastin was also purified from insoluble elastin (purified by procedure A) by the hot alkaline extraction method of Lansing et al. (1952) (procedure B).

Synthesis of allysine-bis-p-cresol derivative

The elastin (100 g) was hydrolyzed in 6N HCl (1L) containing 5% (w/v) *p*-cresol for 48 h at 110°C. One liter of n-hexane/ether (8:2, v/v) was added to the reaction solution, and then the organic layer was discarded. The excess acid and *p*-cresol were removed by rotary evaporation at 50°C. The residue was dissolved with 40 ml of ethyl acetate/acetic

acid/water (1.5:1:1, v/v), and then charged on a large scale silica gel column (130 × 65 mm). Allysine-bis-*p*-cresol derivative (APC) was eluted with 0.5L of ethyl acetate/acetic acid/water (1.5:1:1, v/v), and then evaporated at 50°C. The residue was dissolved with 40 ml of ethyl acetate/acetic acid/water (1.5:1:1, v/v), and then 1 ml of this solution was charged on a LiChroprep Si 60 (40–63 μm) preparative silica gel column (310–25 mm, Merck, Darmstadt, Germany) and ethyl acetate/acetic acid/water (1.5:1:1, v/v) was used as a solvent. The flow rate was 4.0 ml/min. The fractions (20–24 min.) were collected. TLC and analytical HPLC confirmed the purity of APC. TLC was conducted by precoated silica gel 60 on an aluminum sheet (Merck, Darmstadt, Germany) using ethyl acetate/2-propanol/acetic acid/water (4:1:1:1, v/v) as a solvent. Bis-*p*-cresol derivative of allysine revealed strong ninhydrin-positive spot with Rf value of 0.68 (phenylalanine; 0.52). NMR, UV, and FAB-MS characterized this derivative. APC was determined as 2-amino-6,6-bis(2-hydroxy-5-methylphenyl)hexanoic acid as shown in Fig. 1: ^{1}H NMR (DMSOd) δ: 1.237 (1, m, 2H, CH_2), 1.590–1.742 (2, m, 1H, CH_2), 1.839 (3, q, 2H, CH_2), 2.140 (4, d, 6H, CH_3), 3.100 (5, t, 1H, CH), 4.499 (6, t, 1H, CH), 6.635 (7, d, 2H, aromatic proton), 6.737 (8, m, 2H, aromatic proton), 6.868 (9, 2H q, aromatic proton); ^{13}C NMR ((DMSOd)) δ: 20.524 (4, CH_2), 24.169 (1, CH_2), 31.301 (2, CH_2), 33.676 (3, CH_2), 36.212 (6, CH), 54.203 (5, CH), 114.966 (7, aromatic carbon), 126.633 (8, aromatic carbon), 126.748 (10, aromatic carbon), 128.494 (9, aromatic carbon), 130.684 (11, aromatic carbon), 152.520 (12, aromatic carbon), 170.104 (13, COOH); UV λ max (εmax = 6,300 in methanol) 282.2 nm; FAB-MS (*m/z*) 344 ($M + H^+$).

Derivatization of allysine for HPLC analysis

Twenty five milligrams of elastin was precisely weighed and then dissolved in 5 ml of 6N HCl containing 5% (w/v) *p*-cresol in a flame-sealed Pyrex glass. Derivatization of allysine in elastin was carried out under the condition of acid hydrolysis at 110°C for 48 h. Five milliliters of n-hexane/ether (8:2, v/v) was added to the solution after the reaction, and then the organic layer was discarded. Excess *p*-cresol was extracted with n-hexane/ether (8:2, v/v). APC was not detected by HPLC in the organic layer. The resulting solution was evaporated to dryness *in vacuo* at 50°C. The residue was dried over P_2O_5 and dissolved with HPLC solvent (10 ml); then a 20 μl portion of solution was injected into the reverse-phase HPLC column.

Results

HPLC detection of p-cresol derivative

Allysine was labeled with *p*-cresol to obtain APC (Fig. 1). NMR experiments (^{1}H, ^{13}C, ^{1}H–^{1}H COSY, and HSQC) and FAB-MS spectrometry of synthetic *p*-cresol derivative, indicated that 1 mole of allysine reacted with 2 moles of *p*-cresol to yield bis-*p*-cresol derivative, 2-amino-6,6-bis (2-hydroxy-5-methylphenyl) hexanoic acid. The postulated mechanism for the formation of APC is shown in Fig. 2.

The analytical HPLC separation was performed on a reverse-phase C18 column using UV detection at 282 nm, the absorption maximum of APC. Different mobile phase compositions were evaluated, including various combinations of acetonitrile and phosphate buffer at various pHs. The optimum composition was found to consist of 0.05M sodium dihydrogen phosphate buffer (pH 2.2) and acetonitrile. Fine tuning was accomplished by varying the percentage of acetonitrile. Representative chromatograms of

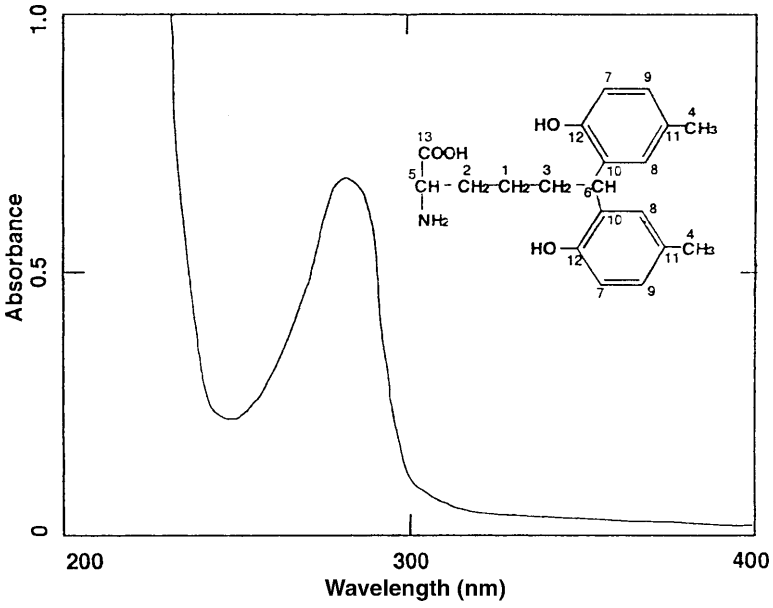


Fig. 1. UV spectra and structure of allysine-*p*-cresol derivative. The *inset* shows the structure of allysine-bis-*p*-cresol derivative

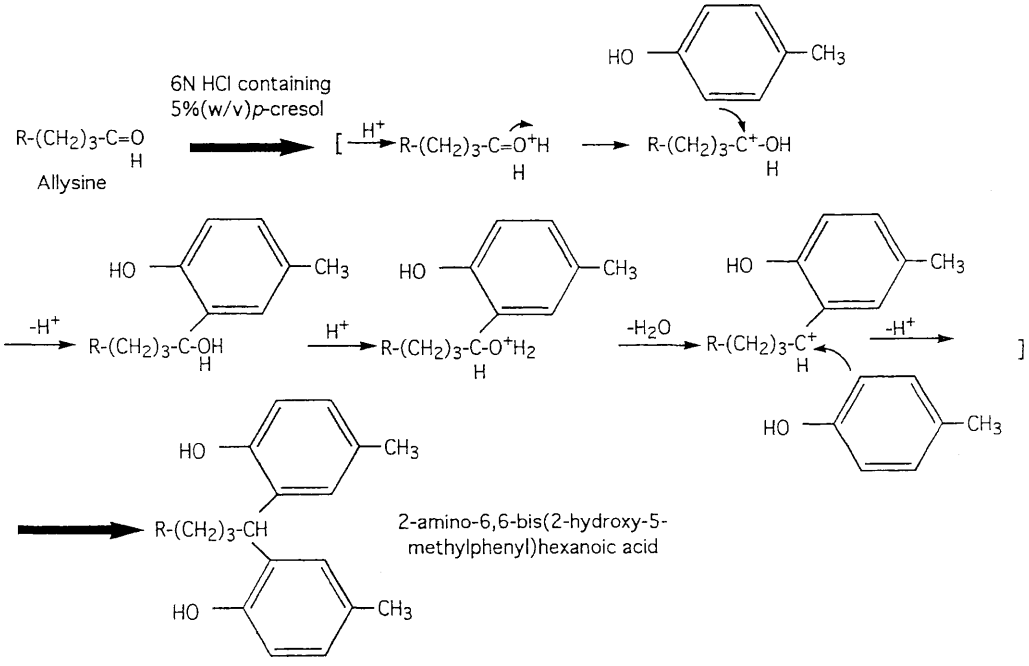
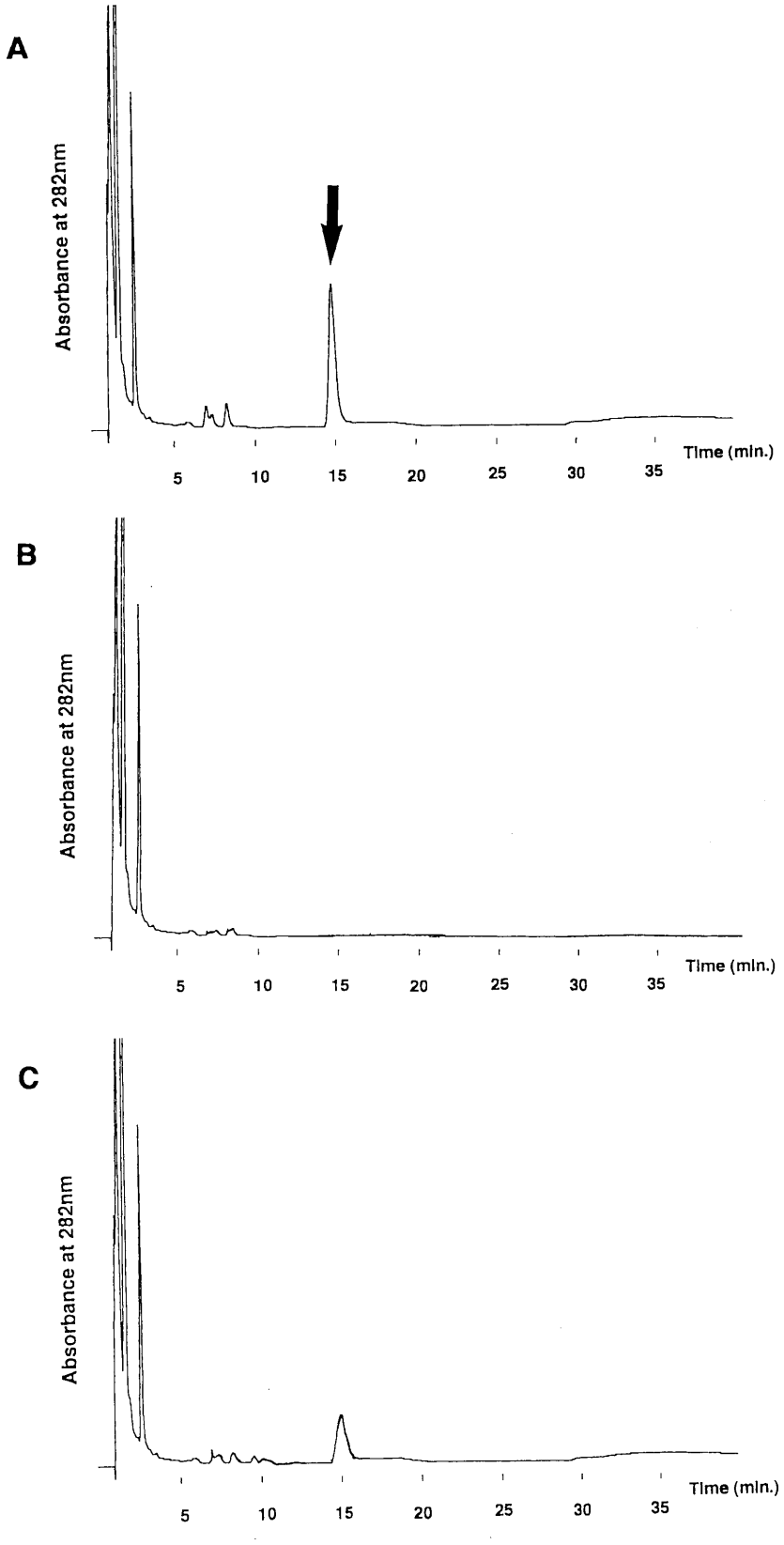


Fig. 2. Derivatization reaction of allysine with *p*-cresol. *R*, side chain of amino acid


A
Absorbance at 282nm
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C
Absorbance at 282nm
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elastin samples from ligamentum nuchae are shown in Fig. 3A. APC showed a single peak and its retention time was about 14.6 min., without interference of other compounds.

Optimization of the derivatization procedure

For derivatization of allysine, elastin was acid hydrolyzed in 6N HCl at 110°C. The optimization of derivatization was studied using 25 mg elastin samples from ligamentum nuchae. The effects of drivatization time (24–72 hr) and of the *p*-cresol concentration (1.0%–10.0%) on the UV response of HPLC were investigated. Fig. 4A shows the UV response as a function of the reaction time using *p*-cresol at a concentration of 5.0% (w/v). The UV intensity reached a plateau for 48 hr and was constant up to 72 hr. This trend was in agreement with the recoveries of desmosine and isodesmosine during acid hydrolysis. Allysine residues in elastin, therefore, were easily derivatized with *p*-cresol, and then were released as APC by acid hydrolysis of elastin. Fig. 4B shows the UV response as a function of the concentration of *p*-cresol using a reaction time of 48 h. The UV intensity maximized and formed at a plateau at 5.0–10.0% of *p*-cresol. As a result, the condition of acid hydrolysis using 6N HCl containing 5.0% (w/v) *p*-cresol (110°C, 48 hr) was finally selected for the derivatization of allysine.

Validation of the method

Quantification of allysine was based on the peak area of APC versus its concentration. The calibration graph, constructed with the amount injected versus peak area of APC, showed a good linearity with the correlation coefficient 0.999 in the determination range 0.222 to 19.623 nmol [Y (peak area) = (7.7937 × 10,000) X (nmol/injection) − 3329.1]. The estimated values for the lower limit of determination and detection were obtained as 115 pmol/injection and 58 pmol/injection at a signal-to-noise ratio of 3, respectively. Satisfactory reproducibility was obtained in the range 5 to 200 mg elastin from bovine ligamentum nuchae; relative standard deviations ranged from 1.1 to 2.5%, as shown in Table 1. A good linear correlation was obtained between the peak area of APC and the dry weight of elastin in the range 5–200 mg [Y (peak area) = 7,310.0 X (mg) − 1921.6, correlation coefficient $\gamma = 1.000$].

Fig. 3. HPLC profiles of acid hydrolysates of bovine ligamentum nuchae elastin, with *p*-cresol (**A**) and without *p*-cresol (**B**), and of bovine ligamentum nuchae elastin reduced with sodium brohydride (**C**). Arrow indicates the peak of APC. Elastin was hydrolyzed in 6N HCl containing 5% (w/v) *p*-cresol for 48 h at 110°C (**A**). Elastin was hydrolyzed in 6N HCl without *p*-cresol for 48 h at 110°C (**B**). After reduction with sodium borohydride (3), reduced elastin was hydrolyzed in 6N HCl containing 5% (w/v) *p*-cresol for 48 h at 110°C (**C**). Analytical HPLC was performed with a LiChrosper 100 RP-18 125-4 reverse-phase column using a solvent of 0.05M phosphate buffer (at pH 2.2)/acetonitrile (3:1, v/v). The flow rate was 1.0 ml/min. Other details are described under "Material and methods"

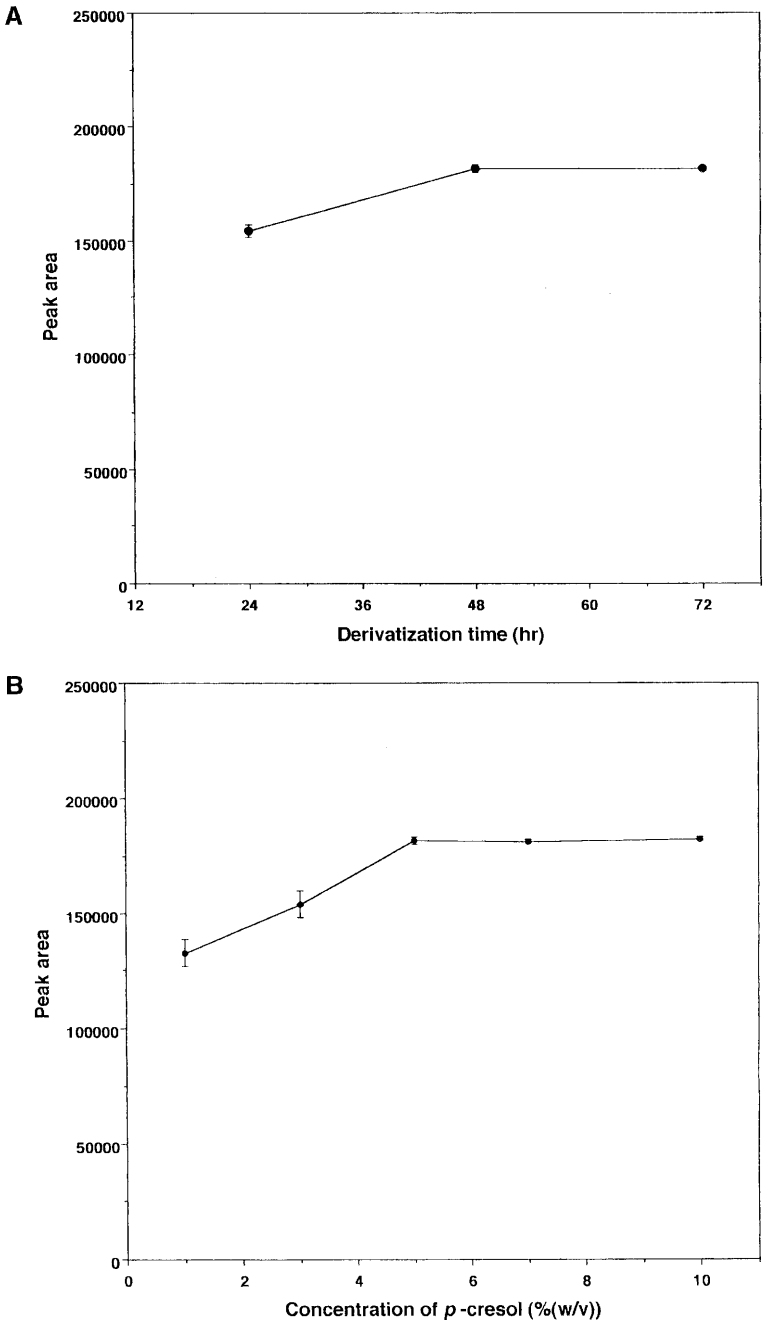

Fig. 4. **A** Effect of hydrolysis time on derivatization of allysine by *p*-cresol. **B** Effect of concentration of *p*-cresol on derivatization of allysine. The elastin from bovine ligamentum nuchae was heated in 6N HCl containing 5% (w/v) *p*-cresol for indicated times (**A**). The elastin from bovine ligamentum nuchae was heated in 6N HCl containing *p*-cresol at indicated concentration for 48 h at 110°C (**B**). Assay for allysine was conducted according to conditions of "Material and methods" and Fig. 3. Values shown are means ± S.D. of three independent determinations

Table 1. Precision of the analytical method for allysine

Elastin* (mg)	Peak area (mean, n = 3)	Allysine level (nmol/mg)	RSD# (%)
5	33,717	47.5 ± 1.0	2.1
10	70,203	47.0 ± 0.7	1.5
25	181,664	47.4 ± 0.5	1.1
50	356,047	46.5 ± 0.7	1.5
100	735,463	47.3 ± 1.2	2.5
150	1,108,710	47.5 ± 1.2	2.5
200	1,448,120	46.1 ± 1.1	2.4

Note: Allysine levels are expressed as nmol per mg of elastin and represents the mean ± S.D. (n = 3). Assay for allysine was conducted according to the conditions in Fig. 3 and "Material and methods".
* Elastin was prepared from ligamentum nuchae by "procedure A" described in "Material and methods". # Relative standard deviation (*RSD*) was used as a measure for precision of the analytical method. RSD is defined as 100 × (S.D./mean).

Table 2. Amount of cross-links in elastin* from different bovine tissues

	Allysine[a]	Desmosine[b]	Isodesmosine[b]
Ligamentum nuchae	47.4	14.2	12.1
Aorta	32.0	12.1	10.1
Lung	10.7	3.3	2.5

Note: Values are expressed as nmol per mg of elastin. * Elastin from bovine tissues was purified by "procedure A" described in "Material and methods."
[a] Assay for allysine was conducted according to conditions of Fig. 3 and "Material and methods".
[b] Desmosine and isodesmosine were quantified by the method of Nakamura and Suyama (1994).

Analysis of allysine in bovine tissue elastin

It has been widely accepted that the elastin was isolated from tissues after other more soluble connective tissue elements were removed, by the use of a harsh method of exposure to hot alkali. The previous reports have shown that the elastin was degraded, as evidenced by a decrease in the level of the desmosines and allysine-aldol, when compared with milder methods (Nakamura and Suyama, 1994; Richmond, 1974). It has also been pointed out that mild procedures should be used for the preparation of the elastin (Paz et al., 1976). In this paper, therefore, we used the elastin (containing polymeric collagen) purified by the mild method with 1M NaCl (procedure A).

Purified elastins from bovine ligamentum nuchae, aorta, and lungs were used to compare the level of allysine with those of desmosine and isodesmosine, as shown in Table 2. These tissues contain high contents of the elastin. That is, the allysine levels in tissues may affect the content of elastin. The distribution also shows two similarities; the content of allysine in these tissues was about three- and four-fold higher than that of desmosine and isodesmosine, respectively.

The presence of 47.4 nmol/mg of allysine in elastin from bovine ligamentum nuchae (purified by procedure A), translates into 59.7 nmol/mg (= 47.4 × 100/79.4) in the elastin (purified by procedure B), since the former is 79.4% of the latter, as previously reported (Nakamura and Suyama, 1994). We also found the presence of 55.0 nmol/mg in the elastin from ligamentum nuchae (purified by procedure B); the alkaline extraction has a destructive effect on the allysine level. The value of 59.7 nmol/mg also translates into 5.0 lysine equivalents per 1,000 amino acid residues. Using the ^{14}[C] NaCN—NH_3 procedure, which has been proved to be reproducible, allysine showed 1.0 lysine equivalents per 1,000 amino acid residues (Pereya et al., 1974). Paz et al. (1976) indicated that the reduction method with $NaBH_4$ (Lent et al., 1969) was not considered to be quantitative. We found that allysine residues in the elastin couldn't be reduced perfectly with $NaBH_4$, as shown in Fig. 3C.

Age-related change of allysine in rat aorta elastin

It is known that desmosine and isodesmosine in aortic and pulmonary elastin, decrease gradually with increasing age (John and Thomas, 1972; Watanabe et al., 1996; Fujimoto, 1982). There are no reports about the age-related changes of allysine in the connective tissue. The allysine residue is a very important precursor for the formation of desmosine and isodesmosine; the precise determination of allysine residue may be a trigger for elucidating the functional changes of elastin.

The allysine content in rat aorta elastin dramatically increased from 1 week to 2 weeks of age, and then was rapidly decreased from 2 weeks to 8 weeks, as seen in Fig. 5. The allysine content was little changed from 8 weeks to 15 weeks. That is, these results show that the turnover of elastin occur most actively before and after the weanling period of rats, and then its turnover occur slowly.

Fornieri et al. (1992) demonstrated that lysyl oxidase activity reached a maximum in about 2 weeks of rat age. Of interest is that the age-related changes of lysyl oxidase activity are similar to that of allysine content.

Discussion

Allysine is an important intermediate compound for the formation of the physiologically crucial cross-linking structure in elastin. Allysine has been identified and determined by derivatization methods using reduction, oxidation, and reaction with CN^- and NH_3 (Lent et al., 1969; Pereya et al., 1974; Miller et al., 1967; Paz et al., 1976). However, since allysine is a highly reactive compound, very little research has been done. These methods have several problems such as complex and time-consuming processes.

In this paper, we report the derivatization of allysine residues in elastin using *p*-cresol as an improved procedure for the analysis of allysine. The allysine-bis-*p*-cresol derivative, 2-amino-6,6-bis(2-hydroxy-5-methylphenyl)hexanoic acid (APC), could be prepared from acid

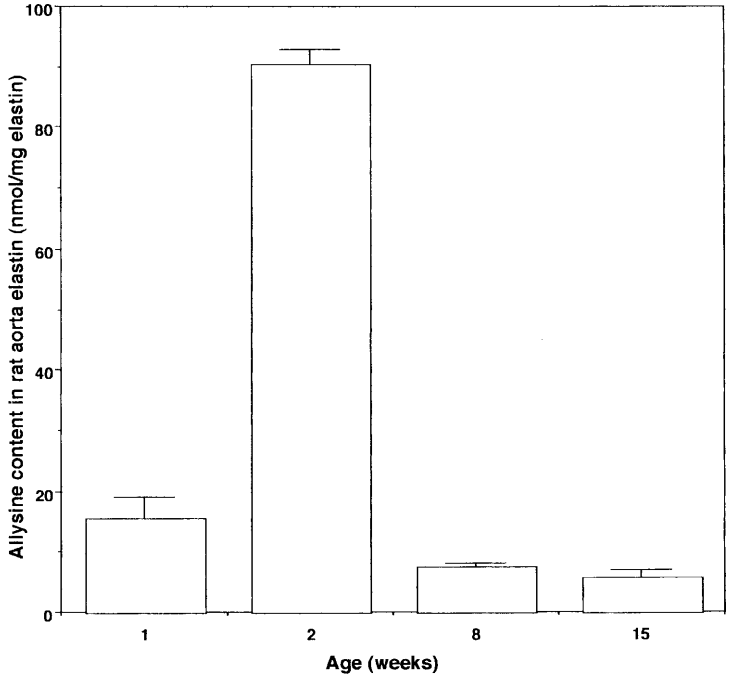

Fig. 5. Change of the allysine content in rat aorta elastin with age. Assay for allysine was conducted according to conditions in Fig. 3A and "Material and methods". Values shown are means ± S.D. of three independent determinations

hydrolysates of elastin using 6N HCl containing *p*-cresol. This *p*-cresol derivative of allysine is the only diaryl product produced from 1 mole of allysine and 2 moles of *p*-cresol, since —OH of *p*-cresol is a more powerful ortho-para-director than its' $—CH_3$. APC can be produced simply through acid hydrolysis similar to ACPP and is stable in the acid solution at 110°C. APC also has good UV absorption; it can be quantitatively determined by the reversed-phase HPLC method with UV detection. From the results of the optimization of the derivatization procedure, the maximum derivatization yields of APC needed the excess of *p*-cresol [5%(w/v)]. This suggests that many unknown molecules containing aldehyde and ketone group exist and the excess of *p*-cresol polymerize without being used for the derivatization of allysine. When the excess of *p*-cresol was extracted with hexane/ether (8:2, v/v), APC showed a sharp single peak in the HPLC chromatogram without the interference of other compounds, as seen in Fig. 3. This method also gave satisfactory recovery and reproducibility for the quantification of allysine in elastin, compared to the previous assays. Our derivatization method will be implemented in current and future investigations of cross-links in elastin.

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