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Bisphenol derivative of allysine for high-performance liquid chromatographic analysis of allysine residue of proteins

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Abstract

Allysine is the most important precursor of physiologically essential cross-links formation in collagen and elastin and is formed by enzymatic oxidative deamination of lysine residues. Because it is a highly reactive aldehyde, many cross-linking amino acid residues may arise from its reaction with other allysine residues or lysine or even histidine residues. We purified and isolated an allysine bisphenol derivative, 1-amino-1-carboxy-5,5-bis-*p*-hydroxyphenylpentane (ACPP), from the reaction products of phenol and allysine residue of bovine ligamentum nuchae by acid hydrolysis in 6 *M* HCl. The structure of ACPP was verified by UV, fast atom bombardment-MS, ^{1}H- and ^{13}C-nuclear magnetic resonance spectroscopies. The optimal reaction condition for ACPP synthesis accompanied by hydrolysis of such proteins was investigated and an ion-paired high-performance liquid chromatographic method for determination of allysine as ACPP was also developed.

Keywords: Allysine; Bisphenol; Proteins

1. Introduction

Connective tissues are ubiquitous in the bodies of animals and have an important role in providing a structural basis for the body and its organs. Collagen and elastin, the most frequently occurring proteins of connective tissues, are stabilized by the formation of covalent bonds in posttranslational processes [1–3]. Collagen and elastin cross-linking are important in many disciplines, as a stabilized extracellular matrix is essential for all animal forms higher than protozoans. Abnormalities of collagen and elastin cross-linking may affect virtually every tissue and organ in the body. Reported abnormalities range from atherosclerosis, emphysema and diabetes mellitus [4–8] to heritable diseases, such as Menkeis disease cutis laxis and forms of Ehlers Danlos syndrome [9].

The cross-linking of elastin and collagen is based on the same fundamental reactions [10–12]. The only known enzyme required for the formation of cross-links in both collagen and elastin is lysyl oxidase (protein lysine 6-oxidase; EC 1.4.3.13) which is a metalloenzyme containing copper [13,14] and it oxidatively deaminates the ε-amino group of lysine residues of such proteins converting to the aldehyde group. This lysine aldehyde is properly called α-amino-adipic-acid-6-semialdehyde, and

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named allysine, as first nominated by Pinnell et al. [15]. Enzymatic formation of allysine from lysine is known to occur in collagen, elastin, eggshell membrane, and the outer membrane of *Escherichia coli* [16–18]. Allysine is one of the most important compounds of the extracellular matrix proteins as an intermediate for the formation of physiologically important cross-linking structure formation. However, because it is a highly reactive compound, very little research has been done on it.

Besides its free form, other possible forms of allysine may exist in extracellular proteins such as hydroxylamine, imine and enamine, hemiacetal and acetal, hemithioacetal and thioacetal, and α,β-unsaturated ether forms. During acid hydrolysis all these forms seem to convert to the free form, allysine, and subjectively decompose to an aldehyde group. Several pairs of allysine in each procollagen and tropoelastin molecule are involved in a set of spontaneous condensation reactions that lead to the formation of the aldimine structures or the aldol [19,20] and other stable quaternary pyridinium cross-linking structures such as desmosine and isodesmosine [21,22], allodesmosine [23] and pentasine [24]. The formation of cross-linking structures is ultimately responsible for the insolubility of the protein.

We prepared a new allysine derivative, which is formed by the Baeyer's reaction theoretically. The derivative is formed by the acid catalyzed condensation of allysine residue and two phenol molecules accompanying hydrolysis, named 1-amino-1-carboxy-5,5-bis-*p*-hydroxyphenylpentane (ACPP). In this paper we describe the isolation and characterization of this new allysine bis-phenol derivative and its analysis method which seems to be a suitable way for studying allysine.

2. Experimental

2.1. Reagents and chemicals

The solvents used for analytical high-performance liquid chromatography (HPLC) were of high quality HPLC grade and of reagent grade for preparative column chromatography, all were obtained from Nacalai Tesque (Kyoto, Japan). Activated charcoal (60–150 mesh) for column chromatography was purchased from Nacalai Tesque and was treated with borohydride in order to avoid oxidation of allysine. Reduction of charcoal with borohydride was done by treatment with 50 g of sodium borohydride and 500 g of charcoal suspended in 1 l of distilled water for 10 h. The reaction was terminated by addition of HCl. Thus obtained reduced charcoal was washed with 3 l of water three times. Silica gel (Art. 7734) for column and silica gel 60 precoated aluminum sheets (Art. 5553) for thin-layer chromatographies were obtained from Merck (Germany).

2.2. Preparation of elastin from bovine ligamentum nuchae

Bovine ligamentum nuchae (about 2 kg) was cleaned from adhering fats, cut into small segments with a blade and then homogenized with 5 l of 1.0 *M* NaCl using a Wareing blender. The blended sample was centrifuged and the precipitate was washed with 5 l of 1.0 *M* NaCl and centrifuged. This treatment was repeated three times and the precipitate obtained was delipidated for 24 h in 5 l of chloroform–methanol (2:1, v/v). After filtration, the delipidated sample elastin was dried over phosphorus pentoxide in vacuo as described previously [25].

2.3. Preparation and isolation of ACPP

A 300-g amount of prepared elastin of bovine ligamentum nuchae was hydrolyzed under nitrogen reflux at 110°C for 48 h in 5 l of 6 *M* HCl in the conventional manner, but in the presence of 3% (v/v) phenol using a 10-l round bottomed flask with a condenser attached. The reaction products containing HCl was evaporated at 60°C to a syrup in a rotary evaporator. The residual syrup was dissolved in 500 ml of distilled water. Thus obtained hydrolysis product of ligamentum elastin was charged on a large-scale borohydride-reduced charcoal column (200×90 mm). The column was washed with water (4 l) to remove hydrochloric acid and most of the neutral amino acids then with 50% methanol (3 l) in order to elute cross-linking amino acids. ACPP was eluted through 100% methanol (2 l). The 100% methanol fraction was evaporated to a syrup, the syrup was dissolved in ethyl acetate–ethanol–acetic

acid–water (7:1:0.2:0.2, v/v) and charged on a silica gel column (170×65 mm) and fractionated using ethyl acetate–ethanol–acetic acid–water (7:1:0.2:0.2, v/v) as the solvent. Fractions of 200 ml each were collected. ACPP-containing fractions, which were monitored by thin-layer chromatography (TLC) were collected and then concentrated in vacuo to a syrup. The white crystal (needle) of ACPP was separated from the syrup by keeping at 4°C overnight then recrystallized from methanol (yield 12 mg). ACPP was eluted before phenylalanine, gave a ninhydrine positive pink spot on silica gel TLC which was conducted on Silica Gel 60 precoated on aluminum sheets (Merck, Art. 5553). The chromatogram was developed by ethyl acetate–ethanol–water (2:1:1, v/v) as the solvent, and each spot on the plate was detected by spraying with 0.2% ninhydrine in 90% ethanol and then heating at 100°C.

2.4. Spectroscopy

Ultraviolet (UV) spectra were recorded with a UV 2100S UV–Visible recording spectrophotometer (Shimadzu, Kyoto, Japan). The sample was dissolved in methanol. Mass spectroscopy (MS) was performed on a JEOL JMS HX-105 mass spectrometer (Japan Spectroscopic, Tokyo, Japan). Ions were generated by fast atom bombardment (FAB), using a xenon primary beam of 70 eV energy. The sample was applied to a matrix consisting of methanol–glycerol (1:1, v/v). ^{1}H-nuclear magnetic resonance (NMR) and ^{13}C-NMR spectra were recorded on a JEOL JNM GSX-270 spectrometer (Japan Spectroscopic) in $CDCl_3$.

2.5. Determination of allysine

Determination of allysine residue as ACPP was carried out by ion-paired HPLC, after a preliminary purification.

2.5.1. Preparation of sample

Sample was cleaned from adhering fats, cut into small segments with a blade and homogenized with 1.0 *M* NaCl as described for elastin preparation. After centrifugation, the residual precipitate was delipidated for 24 h with chloroform–methanol (2:1, v/v). The delipidated sample was dried over phosphorus pentoxide in vacuo. Approximately 10 mg of sample dried over phosphorus pentoxide in vacuo was precisely weighed and then hydrolyzed in a conventional manner for 48 h at 110°C with 5 ml of 6 *M* HCl containing 3% phenol in a Pyrex tube with a PTFE-lined screw cap under nitrogen. The reaction products accompanying hydrolysis were then evaporated to dryness in a rotary evaporator.

2.5.2. Prepurification method

A small Sep-Pak column (Silica cartridges: Art. 20520) obtained from Waters, USA was used for prepurification and the procedure as follows: (1) the Sep-Pak column was flushed with methanol and then ethyl acetate–ethanol–water (5:2:1, v/v). (2) The hydrolysate sample, which was evaporated to dryness, was reconstituted in 500 μl of ethyl acetate–ethanol–water (5:2:1, v/v) and loaded onto the Sep-Pak column. (3) Ethyl acetate–ethanol–water (5:2:1, v/v) was eluted through column, the first 1 ml of eluent was discarded, the next 2.0 ml of eluent containing ACPP was collected carefully and evaporated to dryness, and then reconstituted in 500 μl of distilled water. A 10-μl portion of it was loaded onto the HPLC ODS column.

2.5.3. High-performance liquid chromatography

Analytical HPLC was performed with a reversed-phase Superspher RP-18 ODS column (150×4 mm; Merck). The solvent was 0.1 mol phosphate buffer–acetonitrile (5:1, v/v) containing 20 m*M* sodium dodecyl sulfate (SDS) at pH 3.0. The flow-rate was 1.0 ml/min. The HPLC system consisted of an L-6000 pump, a L-4000 UV detector, and a D-2500 Chromato-Integrator (Hitachi, Tokyo, Japan). The absorbance was monitored at 278 nm. All chromatographic operations were carried out at room temperature.

2.6. Energy calculation

In order to study the minimum energy of ACPP and other possible isomers, we did a thorough analysis using an IRIS INDIGO workstation (Silicon Graphics, USA) running SYBYL software (version 6.1, Tripos, USA), using the Tripos 5.2 Force Field and minimized the energy of ACPP.

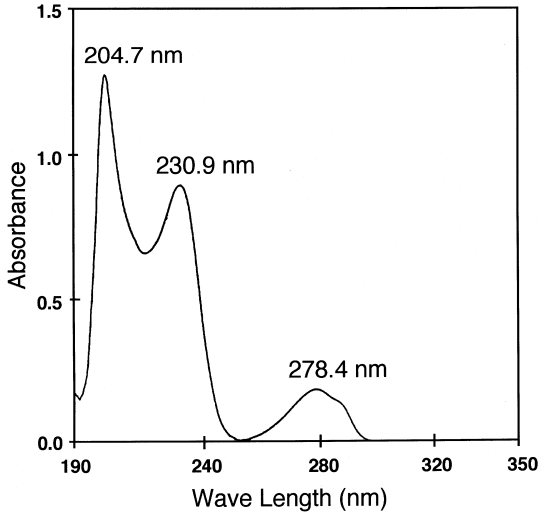


Fig. 1. UV spectrum of ACPP.

3. Results

3.1. Structure of ACPP

ACPP was separated as white crystals (needle, m.p. 156°C, dec.), which were soluble in methanol but not in water. ACPP is a hydrophobic compound and was completely separated from every amino acid residue of protein by TLC as a ninhydrine positive pink spot, which was developed faster than phenylalanine which was developed the fastest of all the amino acids by the solvent system used (ACPP; R_F 0.81, phenylalanine; R_F 0.67). Fig. 1 shows the UV spectrum of ACPP exhibiting two absorption maxima at 230.9 nm and 278.4 nm (MeOH; ϵ_{max}: 3200). The molecular ion region of the positive ion FAB mass spectrum of ACPP is shown in Fig. 2. The spectrum shows a mass of 315 ([M+H$^+$], consistent with an elemental composition of $C_{18}H_{21}NO_4$).

Fig. 3 shows the ^{1}H-NMR and structure of ACPP. δ: 1.18 (m, 2H, C**H**$_2$), 1.79 (m, 2H, C**H**$_2$), 1.83 (m, 2H, C**H**$_2$), 3.68 (t, 1H, C**H**), 3.85 (t, 1H, C**H**), 6.67 (d, 4H, Ar-**H**) and 7.02 (d, 4H, Ar-**H**) were identified. The calculated values for ACPP were close to the predicted values by FAB-MS, with a total of 16 resonating protons. The ^{1}H-NMR of tyrosine was similar to that of ACPP as expected from the close similarity of structure between tyrosine and ACPP. ^{1}H-NMR of tyrosine ($CDCl_3$) δ: 2.64 (m, 2H, C**H**$_2$), 3.85 (t, 1H, C**H**), 6.39 (d, 2H, Ar-**H**), 6.69 (t, 1H, Ar-**H**).

The ^{13}C-NMR spectrum of ACPP, δ: 25.3 (**C**H$_2$), 32.2 (**C**H$_2$), 37.0 (**C**H$_2$), 51.1 (**C**H), 55.5 (**C**H),

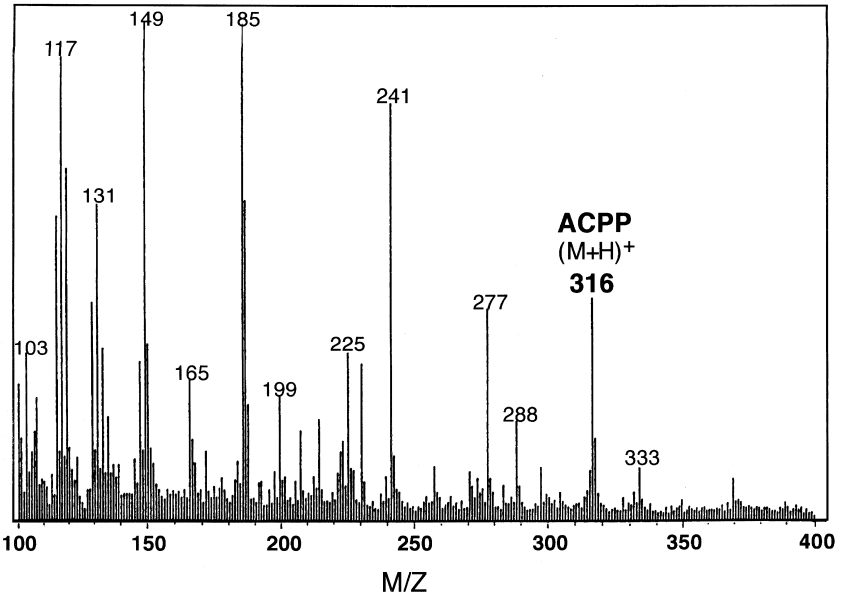


Fig. 2. FAB-MS spectrum of ACPP.

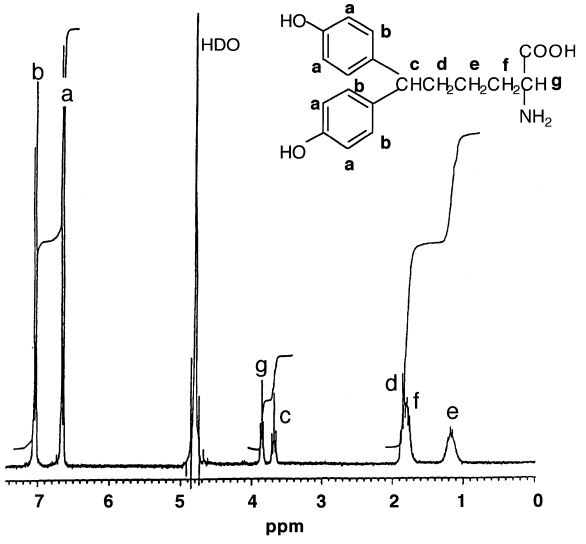


Fig. 3. ^{1}H-NMR spectrum of ACPP.

118.2 (Ar-**C**), 131.6 (Ar-**C**), 133.5(**C**=C) and 173.78 (**C**OOH) were identified.

Distortionless enhancement by polarization transfer (DEPT) C-NMR, ^{1}H,^{1}H-cosy and ^{1}H,^{13}C-cosy spectra (data are not shown) also completely supported the ACPP structure. We proposed it to be a condensation product of one allysine residue and two phenol molecules, 1-amino-1-carboxy-5,5-bis-*p*-hydroxyphenylpentane or α-amino-adipic-acid-6-bis-*p*-hydroxybenzene or bis-*p*-hydroxyphenyllysine.

3.2. HPLC for determination of ACPP

An ion-paired reversed-phase HPLC method was used for the analysis of ACPP. To determine the best solvent system for the separation of ACPP, we examined the effect of the acetonitrile ratio, SDS concentration and eluent pH. A higher acetonitrile rate and SDS concentration resulted in a shorter retention time (t_R) and a lower eluent pH resulted in a longer retention time, considering the position of ACPP being far enough from possible interfering peaks and background. The best solvent was a system of 0.1 *M* sodium phosphate buffer–acetonitrile (5:1, v/v) containing 20 m*M* SDS at pH 3.0. HPLC was done after prepurification as described before. Fig. 4a and b show the HPLC chromatograms of the bovine ligamentum nuchae hydrolysates hydrolyzed with 3% phenol and without phenol, respectively, followed by detection at 278 nm. ACPP was eluted in a t_R of 15.98 min (t_R of tyrosine; 5.94 min) as shown in Fig. 4a. No detectable background peak of ACPP was found in the chromatogram of hydrolysate without phenol as shown in Fig. 4b.

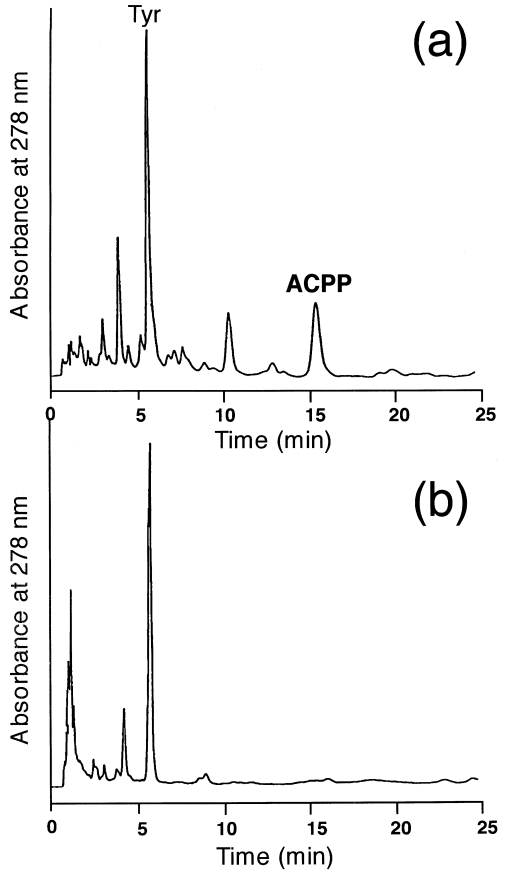


Fig. 4. HPLC of hydrolysate of bovine ligamentum nuchae, (a) with 3% phenol and (b) without phenol. Experimental details given in the text.

3.3. Yield of ACPP

The effect of hydrolysis time on the yield of ACPP was investigated. ACPP was produced very rapidly within 12 h of hydrolysis, reached its highest values within 48 h and remained almost constant thereafter (Fig. 5). Prolonged hydrolysis did not change the ACPP level in bovine ligamentum nuchae. The effect of phenol concentration on ACPP

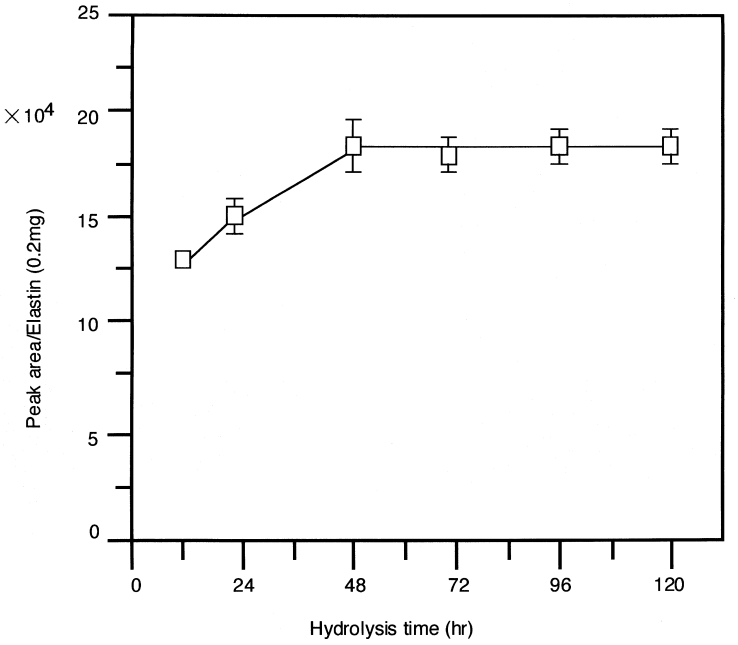


Fig. 5. Effect of reaction time accompanied by hydrolysis of bovine ligamentum nuchae elastin on development of ACPP. Experimental details given in the text.

yield showed no increase in ACPP content by using more than 3% phenol and the study of hydrolysis time and temperature also showed no increase or decrease in ACPP yield at 110°C for 48 h and 120 h, and at 130°C for 48 h.

3.4. Reproducibility

Fifteen samples from the same lot of bovine ligamentum nucae elastin prepared as described above were analyzed under an optimized treatment. Satisfactory reproducibility of ACPP content was obtained, 16.3±0.9 μmol/g elastin as shown in Fig. 6.

3.5. Energy calculation

From the results of not only TLC, HPLC but also ^{1}H- and ^{13}C-NMR we obtained only ACPP as the bis-*p*-hydroxyphenyl derivative of allysine, but theoretically there should be other possible isomers of ACPP, as two isomers of bis-*o*-hydroxyphenyl derivative and *p*-*o*-hydroxyphenyl derivative of allysine, but actually we could not obtain such isomers here. In order to study such possibilities (bis-*o*- and *p*-*o*-), we compared the energy of isomers. The energy computation results of seven isomers (bis-*para*, two bis-*ortho*, four *para*-*ortho*) are shown in Table 1. The total energy for ACPP was −28.950 kcal/mol, which was not different from the other isomers as shown in Table 1. However, ACPP has the lowest bond stretching, torsional,

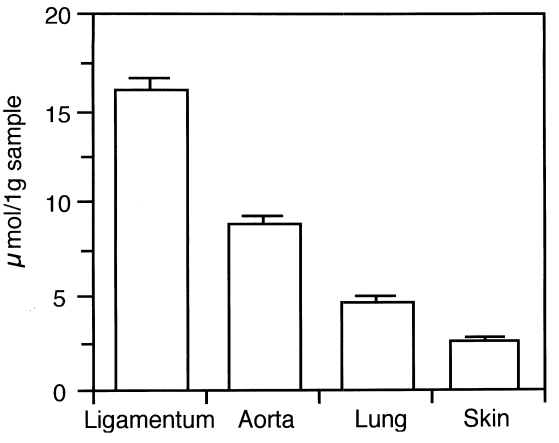
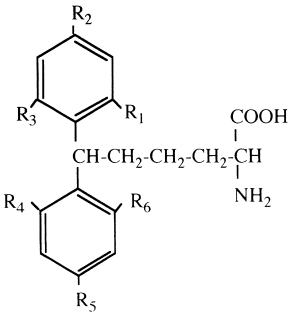


Fig. 6. ACPP (allysine) content of bovine tissues.

Table 1
Minimum energy of ACPP and seven isomers



Bis-*para*	R_1, R_3, R_4, R_6: H, R_2, R_5: OH
Bis-*ortho* 1	R_1, R_2, R_4, R_5: H, R_3, R_6: OH
Bis-*ortho* 2	R_2, R_3, R_5, R_6: H, R_1, R_4: OH
para-ortho R1	R_1, R_3, R_5, R_6: H, R_2, R_4: OH
para-ortho R2	R_1, R_3, R_4, R_5: H, R_2, R_6: OH
para-ortho S1	R_1, R_2, R_4, R_6: H, R_3, R_5: OH
para-ortho S2	R_2, R_3, R_4, R_6: H, R_1, R_5: OH

	Bis-phenol derivatives of allysine						
	Bis-*para*	Bis-*ortho* 1	Bis-*ortho* 2	*para-ortho* R1	*para-ortho* R2	*para-ortho* S1	*para-ortho* S2
Bond stretching	0.760	0.870	0.847	0.826	0.818	0.812	0.905
Angle bending	5.760	5.980	5.660	6.232	5.939	6.196	6.948
Torsional	1.423	2.106	1.544	1.754	2.984	1.898	1.532
Out of plane bending	0.002	0.004	0.005	0.002	0.003	0.003	0.001
1–4 Van der Waals	8.071	8.276	8.225	8.020	7.799	8.029	8.185
Van der Waals	−3.619	−3.847	−3.693	−5.133	−4.725	−5.093	−4.393
1–4 Electrostatic	−12.613	−10.852	−10.646	−11.997	−11.675	−11.755	−11.851
Electrostatic	−28.732	−29.920	−29.678	−27.997	−33.064	−28.402	−28.687
Total	−28.950	−27.384	−27.736	−28.293	−31.922	−28.313	−27.359

and Van der Waals energies among the seven isomers. Especially, the torsional energy of ACPP (1.423 kcal/mol) was considerably lower than that of the *para-ortho* R2 isomer (2.984 kcal/mol).

3.6. ACPP content of various bovine tissues

We determined the ACPP content of various bovine tissues such as ligamentum nuchae ($n=15$), aorta ($n=3$), lung ($n=3$) and skin (regio collidorsalis, $n=3$). The results are shown in Fig. 6. Bovine ligamentum nuchae showed the highest ACPP content among the bovine tissues determined. Ligamentum nuchae contained 16.3 μmol/g of ACPP that was approximately two-times the content in the aorta and four-times that in the lungs and skin.

The effect of the hot alkaline procedure, which is usually used for the separation of insoluble elastin from polymeric collagen, was investigated. Hot alkali treatment (20 volumes of 0.1 *M* NaOH heated with stirring in a boiling-water bath for 10 min as the usual method) of ligamentum nuchae elastin prepared in this experiment described above decreased the amount of ACPP by about 30%. This showed that allysine is not completely labile under a hot alkaline treatment.

4. Discussion

While allysine is a very important precursor of construction of cross-links in both elastin and collagen and is a highly reactive compound, we searched for a proper allysine derivative, which can be used in quantification of the allysine residue in proteins. Here, we have described the quantitative preparation method of a new allysine derivative accompanying the acid hydrolysis of collagen and elastin. We obtained a new allysine derivative named 1-amino-1-carboxy-5,5-bis-*p*-hydroxyphenylpentane (abbreviated as ACPP). ACPP which is a phenol derivative of allysine and can be produced simply through acid hydrolysis. From the structure identification, we concluded that ACPP is the condensation

product of one allysine residue and two phenol molecules that is bis-*p*-phenol derivative of allysine. We also tried other compounds instead of phenol, such as resorcinol, phloroglucinol and dimedone, which seemed to have the possibility of producing other isomers, but they also produced brown polymers, and we could not obtain a stable ACPP structure by treatment with 6 *M* HCl at 110°C for 48 h hydrolysis. The production of ACPP and not other isomers is because of bond stretching, torsional and Van der Waals energies being the lowest, and it is also probably because of a steric hindrance effect in nucleophilic substitution. It is well known that bisphenol A [2,2-bis-(4′-hydroxyphenyl)-propane] is the only compound formed by the condensation reaction of acetone and phenol in the presence of acid catalysis. The reaction supported the formation of ACPP and not other isomers.

Here, we developed a method for quantification of allysine residue as ACPP in elastin and collagen by HPLC. The method used and the results achieved in this study should be useful for further investigations on other unknown aspects of allysine.

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