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# Antioxidative Collagen-Derived Peptides In Human-Placenta Extract

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Human-placenta extract (PLx), has been widely used in clinical and cosmetic fields, and is known to possess marked antioxidant activity. We have attempted to identify the antioxidative components in PLx. Initially, we purified PLx using Sephadex G-10 column. The first eluted peak which had optical density at 280 nm and exhibited approximately 20 per cent of antioxidant activity of the applied PLx, did not contain uracil, tyrosine, phenylalanine, or tryptophan which are the previously identified antioxidants from PLx. This fraction was further purified by reverse-phase high performance liquid chromatography. All eluted peaks containing antioxidant activity exhibited optical density at 280 nm. Six separate fractions of eluent having antioxidant activity were analyzed by an amino acid sequencer, and each turned out to contain Glycine(G)-XY amino acid repeats, which appear to be derived from collagen. These results suggest that peptides produced from collagen are also antioxidative components of PLx.
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## INTRODUCTION

Exposure of pregnant females to several xenobiotics frequently causes embryonic death, fetal malformations or birth defects (Halliwell and Gutteridge, 1999). Oxidative stress has been suggested to contribute to the action of several teratogens in whole or in part (Wells and Winn, 1996). Therefore, human placenta contains antioxidative defences to protect embryo from oxidative stress (Avissar et al., 1994; Thomas et al., 1998; Kankofer, 2001). Human placenta extract (PLx) prepared from the placenta of healthy pregnant females is known to have various physiological actions, including antioxidative properties (Mochizuki and Kada, 1982; Banerjee et al., 1992; Cronin and McFadden, 1993). Since PLx has been known to have no toxic effects, it appears that PLx or its antioxidants can be used as therapeutic agents for other oxidative stress-related diseases, such as atherosclerosis and diabetes.

In order to clarify the antioxidative properties of PLx, previously we purified the antioxidative components from PLx. Uracil, tyrosine, phenylalanine, and tryptophan, were shown to account for approximately 59 per cent of the antioxidant activity of PLx (Togashi et al., 2000a). Then we indicated the suppressive effects of the purified antioxidants on acute ethanol-induced liver injury in mice (Togashi et al., 2000b). However, preliminary investigations revealed that PLx contains macromolecular antioxidants, which account for considerable antioxidant activity of PLx. In this study, we identify collagen peptides as new antioxidants distinct from uracil, tyrosine, phenylalanine, and tryptophan in PLx.

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## MATERIALS AND METHODS

### PLx and chemicals

Lyophilized PLx used for the experiments was prepared by Snowden Co. Ltd (Tokyo, Japan) for commercial use. According to the information from Snowden Co. Ltd, PLx was prepared by hydrolysing human placenta with proteases after being homogenized in a physiological salt solution. The hydrolysate was stored for one month at 4°C in the dark, then centrifuged, and the supernatant was lyophilized. The resulting powder was dissolved in distilled water at a concentration of 10 per cent (10 per cent crude PLx).

Hydrogen peroxide (30 per cent aqueous solution) was purchased from Kanto Reagents (Tokyo, Japan). Ammonium iron (II) sulphate hexahydrate, 2-deoxy-D-ribose, trichloroacetic acid, and 4,6-dihydroxy-2-mercaptopyrimidine (thiobarbituric acid) were purchased from Wako Pure Chemical Industries Ltd (Osaka, Japan). Collagen type IV derived from human placenta was obtained from Sigma Chemical Co. (St Louis, MO, USA). *Clostridium histolyticum* collagenase (type I) was purchased from Worthington Biochem. Co. (Lakewood, NJ, USA). All other reagents were of HPLC or analytical reagent grades.

### Assay of antioxidant activity

Antioxidant activity was determined by the deoxyribose method as described by Rice-Evans et al. (1991). Briefly, to a

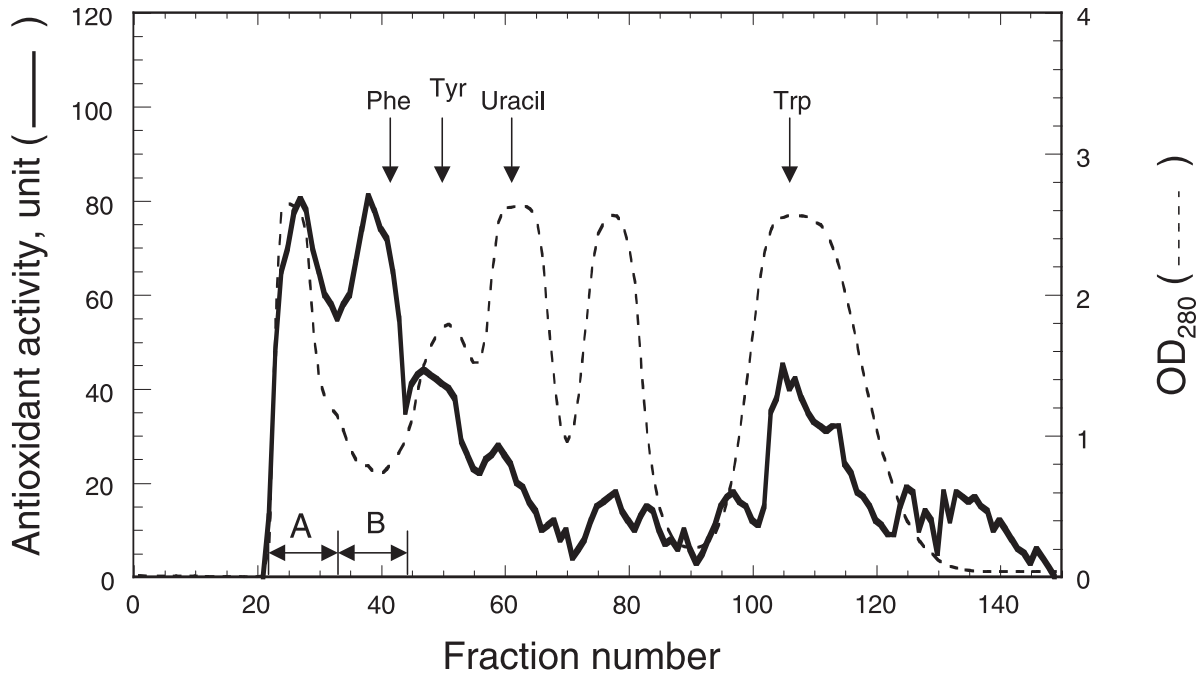


**Figure 1.** Sephadex G-10 chromatography of PLx. PLx was subjected to Sephadex G-10 chromatography: column size; i.d. 30 × 824 mm (582 $cm^3$), eluent; $H_2O$, flow rate; 1 ml/min; sample volume; 30 ml, and 10 ml/fraction. UV absorbance (280 nm) (– – –) of each fraction was measured using a Hitachi U-2000 spectrometer. Antioxidant activity (——) is expressed as μmol equivalents of mannitol by the deoxyribose method.

reaction mixture containing 0.5 ml of 150 mM NaCl, 0.2 ml of 5 mM deoxyribose, 0.15 ml of sample, 0.1 ml of 2 mM ferrous ammonium sulphate, and 0.05 ml of 0.03 per cent (v/v) $H_2O_2$, 0.5 ml of 1 per cent (w/v) thiobarbituric acid (TBA) in 0.05 M NaOH and 0.5 ml of 2.8 per cent (w/v) trichloroacetic acid were added. The mixture was heated for 15 min at 100°C, and cooled, and absorbance at 535 nm was measured. One unit of antioxidant activity was defined as the amount of antioxidant which leads to the same value of $\Delta OD_{535}$ obtained by a 1 μmol dose of mannitol.

### Purification of antioxidants in PLx

PLx (30 ml) was applied to a Sephadex G-10 column (i.d. 30 × 824 mm, 582 ml) pre-equibrated with distilled water, and eluted with distilled water at a flow rate of 0.4 ml/min. The eluted fractions (10 ml each) were assayed for antioxidant activity, and active fractions (zone A and B in Figure 1) were collected, lyophilized, and dissolved in 30 ml of distilled water.

Reverse-phase (RP)-HPLC was performed on a DELTA 3000 apparatus (Waters, USA) equipped with an UV detector operated at 280 nm. Two ml of each sample was applied to an ODS Mightysil RP-18 GP column (5 μ particle size, i.d. 10 × 250 mm, 19.6 ml, Cica MERCK, Japan) and eluted with an acetonitrile/water linear gradient system (5–30 per cent acetonitrile for 250 min) at a flow rate of 1.0 ml/min.

### Identification of antioxidants purified from PLx

Amino acid sequences of peptide fragments were performed by automated Edman degradation using an amino acid sequencer Procise cLC (PE Applied Biosystems, Foster City, CA, USA). Amino acid sequences were searched in BLAST sequence data.

### Electrophoresis and molecular weight determination

Polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulphate (SDS) was performed on slab gels (13 per cent polyacrylamide) as described by Laemmli (Laemmli, 1970), and protein was stained with a silver staining kit (Wako Pure Chemical Industries Ltd). An electrophoresis calibration kit (Amersham Pharmacia Biotech), composed of phosphorylase b (94 000), bovine serum albumin (67 000), ovalbumin (43 000), carbonic anhydrase (30 000), soybean trypsin inhibitor (20 100) and α-lactalbumin (14 400), was used as molecular weight standards.

## RESULTS

### Separation of antioxidant by Sephadex G-10 column chromatography

PLx containing 3100 units of antioxidant activity was subjected to Sephadex G-10 column chromatography. This chromatography was not based on gel filtration but adsorption, and the sharp separation provided by this chromatography was essential for purification of antioxidants in PLx. Elution profiles of antioxidant activity and absorbance at 280 nm are shown in Figure 1. Four major peaks exhibiting antioxidant activities are

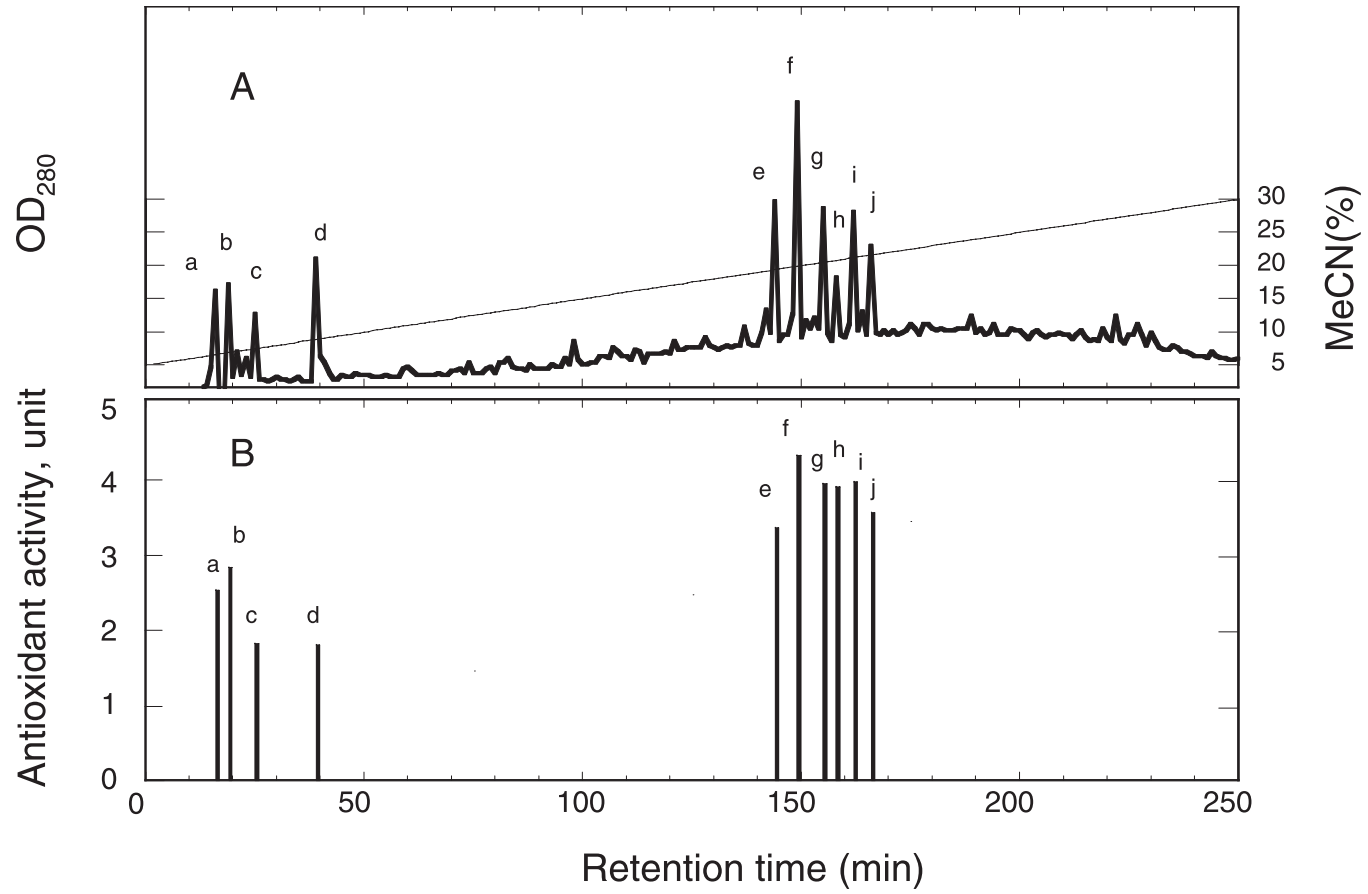


**Figure 2.** Separation of antioxidants in PLx by HPLC (A) and their antioxidant activity (B). Sample derived from zone A fraction in Figure 1 was subjected to RP-HPLC: column; ODS Mightysil RP-18 GP (5 μ), column size; i.d. 10 × 250 mm (19.6 $cm^3$), eluent; linear gradient of acetonitrile in $H_2O$ [5–30% (v/v)] containing 0.1% (v/v) trifluoroacetic acid, flow rate; 1.0 ml/min for 250 min, sample volume; 2 ml. Antioxidant activity is expressed as μmol equivalent of mannitol by the deoxyribose method.

seen. Previously identified phenylalanine, tyrosine, uracil, and tryptophan, were eluted in fractions after the second peak (Figure 1). Recoveries of antioxidant activity from the first peak (zone A: fraction 23–31) and the second peak (zone B: fraction 32–44) were approximately 20 per cent (629 units) and 21 per cent (642 units), respectively. Zone A had peak absorbance at 280 nm, but not zone B.

## Purification of antioxidant by RP-HPLC

Zone A was collected, and an aliquot (70 units of antioxidants) was purified further by RP-HPLC using an acetonitrile linear gradient (5–30 per cent acetonitrile, 250 min). As shown in Figure 2A, all ten major peaks (peak *a–j*) had absorbance at 280 nm, exhibited antioxidant activity (Figure 2B). Retention times and acetonitrile concentrations were 144 min and 20 per cent for *e*, 149 min and 21 per cent for *f*, 154 min and 21 per cent for *g*, 158 min and 21 per cent for *h*, 162 min and 22 per cent for *i*, and 166 min and 22 per cent for *j*. Total antioxidant activity of the combined peaks *a–j* was approximately 50 per cent (35 units) of the sample applied to the HPLC column.

## Identification of antioxidants in PLx

Combined peaks *e–j*, which represent 72 per cent of antioxidant activity of combined peaks *a–j*, were subjected to amino acid sequence analysis. As shown in Table 1, partial sequences of all peptides contained repeats of the amino acid sequence motif, GXY, where X and/or Y is frequently proline (Pro) or hydroxyproline (Hyp). Computer search of BLAST sequence data bases indicated that peak *e* was a partial sequence of α4 Type IV collagen; peak *f*, α2 Type IV collagen; peak *g*, α1 Type II collagen; peak *h*, α2 Type IV collagen; peak *i*, Type IV collagen; and peak *j*, α1 Type V collagen (Table 1). The molecular weight range of these six peptides as estimated by SDS-polyacrylamide gel electrophoresis was approximately from 25 kDa to 43 kDa (data not shown). These results indicated that antioxidants *e–j* all were collagen peptides.

## Effect of incubation on antioxidant activity in PLx

Next, PLx was incubated either at 30°C or at −20°C for 20 days and change of its activity was investigated. As shown in

**Table 1.** Amino acid sequence of antioxidants (peaks *e*, *f*, *g*, *h*, *i*, and *k*) separated by HPLC

| Sample | Residue 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 | 16 | Homologue |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Peak *e* | G | I | Hyp | G | V | Hyp | G | H | A | G | E | Y | G | A | Hyp | G | Type IV collagen |
| Peak *f* | G | L | Hyp | G | Q | I | G | A | Hyp | G | A | Hyp | G | L | A | G | Type IV collagen |
| Peak *g* | G | A | Hyp | G | F | Hyp | G | N | A | G | A | Hyp | G | P | A | G | Type II collagen |
| Peak *h* | G | L | Hyp | G | K | Hyp | G | A | Hyp | G | A | Hyp | G | P | A | G | Type IV collagen |
| Peak *i* | G | D | K | G | N | V | K | A | Hyp | G | V | K | G | P | A | G | Type IV collagen |
| Peak *j* | G | P | Hyp | G | P | Q | G | A | Hyp | G | E | Hyp | G | P | Hyp | G | Type V collagen |

Amino acid sequences of antioxidants (peaks *e*, *f*, *g*, *h*, *i*, and *k*) separated by HPLC (Figure 2) were determined by protein microsequencing as described under 'Materials and Methods'. Sequences obtained in this fashion were searched in BLAST database by the computer.

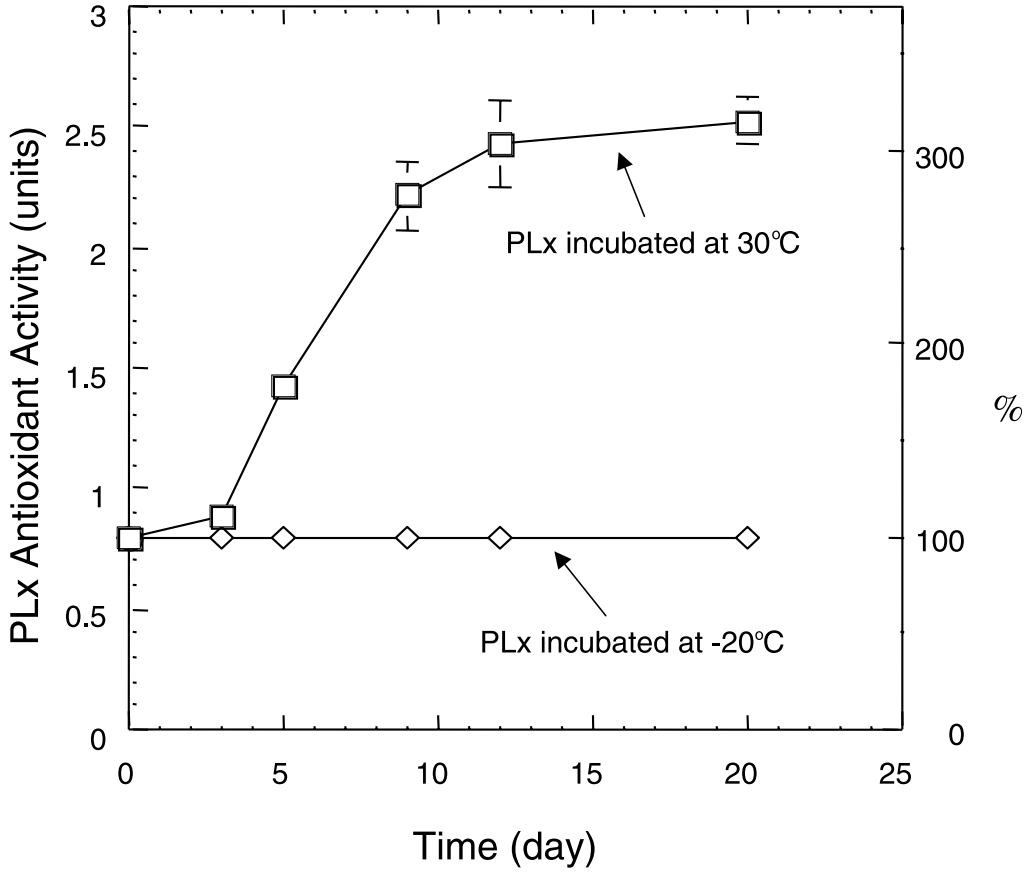


**Figure 3.** Increase in antioxidant activity of PLx by incubation. PLx containing 0.8 units of antioxidant activity was incubated either at 30°C (□) or at −20°C (◇), and at the time indicated, antioxidant activity of the PLx was determined by the deoxyribose method as described in 'Materials and Methods'. Antioxidant activity is expressed as μmol equivalents of mannitol. Error bars indicated s.d. for triplicate determinations.

Figure 3, antioxidant activity in PLx incubated at 30°C for 20 days was approximately 3.2-fold higher than the original activity, while the incubation at −20°C did not cause any change in antioxidant activity. These results indicated that chemical breakdown and/or hydrolysis by proteases of components in PLx increase antioxidant. In addition, PLxs incubated at 30°C for 2–5 days were purified by RP-HPLC using the same conditions as described above. Peaks exhibiting absorbance at 280 nm and exhibiting antioxidant activities were subjected to amino acid sequence analysis. These antioxidants were shown to be collagen peptides (data not shown).

## Antioxidant activity of collagen hydrolysates

In order to confirm the involvement of collagen hydrolysates in the antioxidant activity of PLx, we measured antioxidant

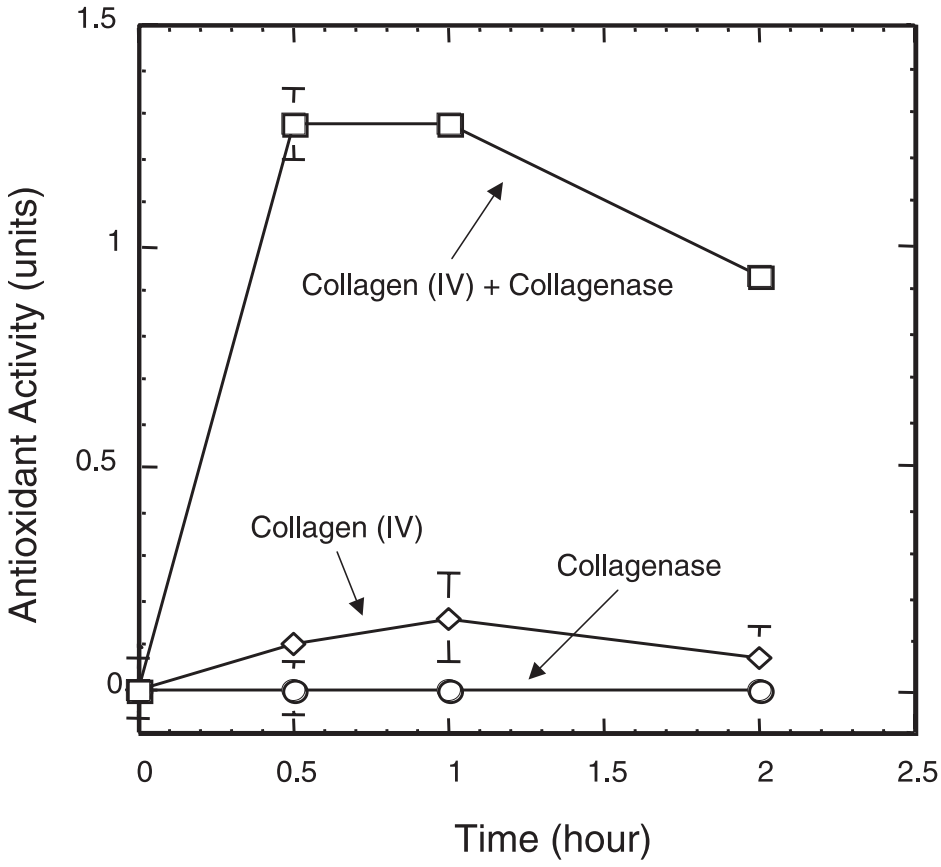


**Figure 4.** Effect of collagenase treatment on antioxidant activity of collagen (IV). Type IV collagen (0.4 nmol) alone (◇), *Clostridium histolyticum* collagenase (0.05 nmol) alone (○), and Type IV collagen (0.4 nmol) in the presence of *Clostridium histolyticum* collagenase (0.05 nmol) (□) were incubated at 37°C at pH 7.0, and at the time indicated, antioxidant activity of the reaction mixture was determined by the deoxyribose method as described in 'Materials and Methods'. Antioxidant activity is expressed as μmol equivalents of mannitol. Error bars indicated s.d. for triplicate determinations.

activity in hydrolysates of commercially available Type IV collagens purified from human placenta. As shown in Figure 4, antioxidant activity of Type IV collagen in the presence of *Clostridium histolyticum* collagenase increased and reached 18-fold of the original activity after 2 h of incubation. These results suggest that peptides having antioxidant activity are generated from type IV collagen by proteolytic hydrolysis.

## DISCUSSION

In this study, we have purified a new type of antioxidants from PLx, and have identified them to be collagen peptides. The antioxidant activity of these collagen peptides accounted for approximately 15 per cent of the total antioxidant activity of PLx.

Human placenta contains many kinds of collagen, including type I, II, III, IV, V, VII, VIII, and XVI (Niyibizi et al., 1984; Fujimoto, 1997; Kypreos et al., 2000). Collagens maintains the moisture within skin. Previous studies have shown that collagens are involved in oxidative processes (Hawkins and Davies, 1997). Reactive oxygen species inhibit collagen synthesis in a variety of noncardiac cell types, including rat lung, dermal fibroblasts, and human venous endothelial cells (Belkhiri et al., 1997). On the other hand, ascorbic acid, which has a well documented role in collagen metabolism as a direct requirement for prolyl and lysyl hydroxylases, stimulates collagen production in cultured chick tendon and human skin fibroblasts (Kao et al., 1976, 1977). Mario et al. have reported that ascorbic acid induces lipid peroxidation and the production of reactive aldehydes, and that this may be essential for stimulation of collagen gene expression by ascorbic acid in cultured human fibroblasts (Chojkier et al., 1989).

In this study, we first have shown that collagen peptides exhibit antioxidant activity. While further studies are required to clarify the mechanism of antioxidative action of collagen peptides, the oxidation of collagen-derived peptides has been reported in detail (Hawkins and Davies, 1997). Hydroxyl radicals produced from a $Fe(II)/H_2O_2$ redox couple attack both side chains (–CHR′R″) (Pro/Hyp) and α-carbons [–C(R)($NH^{3+}$)COO–, R=side chain] (Pro/Hyp) on collagen peptides (Hawkins and Davies, 1997). In addition, hydroxyl radicals attack the free amino acids, Pro and Hyp, particularly at the C-3 position of Pro and at the side chain of Hyp, respectively. Collagen is characterized by a triple helical structure with a repeating sequence of Gly-X-Y, where amino acids X and Y are with high frequency Pro and Hyp, respectively. Tri-amino acid sequences, Gly-Pro-Y and

Gly-X-Hyp occur in 21.4 per cent and 21.2 per cent, respectively, of total α1 chain triplets (Gross, 1974). Gly-Pro-Hyp accounts for 10.1 per cent of triplets (Gross, 1974). It is possible that the oxidation of Pro and Hyp on collagen is involved in the antioxidant activity of collagen peptides. The mechanism of the increase in the oxidative activity of collagen by collagenase treatment is under investigation.

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