Purification and Identification of Antioxidant Substances in Human-Placenta Extracts

Shin-ichi Togashi, Noriko Takahashi,* Yoshinori Kubo, Atsushi Shigihara, Kimio Higashiyama, Satoshi Watanabe and Tetsuya Fukui

Department of Health Chemistry, Faculty of Pharmaceutical Sciences, Hoshi University, Shinagawa-ku, Tokyo 142–8501, Japan

(Received October 19, 1999; Accepted November 18, 1999)

We attempted to purify and identify the active antioxidants in human-placenta extracts (PLx). Purification was performed successively by 40% methanol precipitation, Sephadex G-50 gel chromatography, and high-performance liquid chromatography (HPLC). The supernatant of PLx after 40% methanol precipitation possessed 85% of the original antioxidant activity. Elution of the antioxidants from gel chromatography gave mainly three peaks with approximately 98% recovery of the antioxidant activity. The antioxidant activity was then fractionated by HPLC with a recovery of approximately 90% of the activity. Three components were obtained, and based on HPLC retention times, UV, NMR, and FAB-MASS, they were identified as uracil, tyrosine, and phenylalanine.

Key words —— placenta, antioxidant, uracil, tyrosine, phenylalanine, identification

INTRODUCTION

Oxidative stress occurs in aerobic cells or tissues when the amount of generated reactive oxygen species (ROS) exceeds the antioxidant capacity of the target cell. ROS may interact with and modify cellular protein, lipid, and DNA, resulting in altered target cell functions. The accumulation of oxidative damage has been implicated in both acute and chronic cell injuries. Especially, recent information indicates that uncontrolled lipid peroxidation induced by increased ROS may contribute to certain disease processes *via* disruption of membrane lipids and other cell components.[1,2]

Living cells have defensive mechanisms to protect vital cell components against ROS. The primary components of the physiological antioxidative system are the superoxide dismutase (SOD), catalase and glutathione (GSH). SOD rapidly dismutates superoxide anions to H_2O_2 and O_2 in the presence of H^+. Decomposition of H_2O_2 is mainly carried out by either catalase or by the coupled glutathione peroxidase (GSH-Px) and glutathione reductase (GSSG-Rd) enzyme system at the expense of reduced GSH.

Recent reports elucidate the potential significance of antioxidant supplementation[3–5] in the management of reactive oxygen-mediated oxidative stress, known to be involved in the development and exacerbation of several degenerative diseases such as artery hardening, diabetes, nervous disorders, and many other diseases and aging.[6]

Human-placenta extracts (PLx) have been reported to possess antioxidant activity[6] and are widely used in clinical and cosmetic fields due to effects on inhibition of melanin formation, moisture retention, and keratolytic and anti-inflammatory actions.[7–12] The antioxidants in PLx may greatly contribute to prevention or cure of many diseases triggered by reactive oxygen. Although PLx is known to contain various enzymes, vitamins, amino acids and nucleic acids,[13,14] the main substances contributing to the antioxidant activity of PLx have not been elucidated. In this study, we attempted to purify and identify the antioxidants in PLx.

*To whom correspondance should be addressed: Department of Health Chemistry, Faculty of Pharmaceutical Sciences, Hoshi University, 4–41, Ebara 2-Chome, Shinagawa-ku, Tokyo 142–8501, Japan. Tel.: +81-3-5498-5950; Fax: +81-3-3787-0036; E-mail: t-noriko@hoshi.ac.jp.

MATERIALS AND METHODS

Reagents and Chemicals —— Lyophilized PLx for commercial use was provided from Snowden Co. Ltd. (Tokyo, Japan) and used for the experiment. According to the information from Snowden Co. Ltd., PLx was prepared by hydrolyzing the human-placenta with certain proteases after being homogenized in a physiological salt solution, and stored for one month at 4℃ in the dark. The supernatant after centrifugation was collected, treated to remove lipophilic substances, such as estrogen whose derivatives are known to show antioxidant activity, and lyophilized.[15] The resulting powder was dissolved in distilled water at a concentration of 10% (10% crude PLx).

Hydrogen peroxide (30% aqueous solution) was purchased from Kanto Reagents (Tokyo, Japan), and ammonium iron (II) sulfate 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). All other reagents used in this study were of HPLC or analytical reagent grades.

Measurement of Antioxidant Activity —— Conjugated dienes method: The conjugated dienes method was performed basically as described by Kharasch and Novak.[16] Briefly, linoleic acid (24 mg) was added to a solution of 10 ml of 30 mM NaCl and 10 ml of 0.8% sodium dodecyl sulfate. The mixture was sonicated and further mixed with 60 ml of 30 mM NaCl (linoleic acid suspension). The reaction mixture containing 2.5 ml of the linoleic acid suspension, 0.1 ml of sample, 50 μl of 0.003% H_2O_2, and 25 μl of 1% ferrous ammonium sulfate was kept in a cuvette with a 1-cm light path at 25℃ under continuous stirring. The rate of absorbance at 234 nm (ΔOD_{234}/min) after the addition of ferrous ammonium sulfate was monitored by spectrophotometer (Fig. 1A).

Deoxyribose method: The deoxyribose method was performed as described previously by Rice-Evans *et al.*[17] The reaction solution containing 0.5 ml of 150 mM NaCl, 0.2 ml of 5 mM deoxyribose, 0.15 ml of H_2O (control) or sample, 0.1 ml of 2 mM ferrous ammonium sulfate, and 0.05 ml of 0.03% (v/v) H_2O_2 was mixed. Then, 0.5 ml of 1% (w/v) thiobarbituric acid (TBA) in 0.05 M NaOH and 0.5 ml of 2.8% (w/v) trichloroacetic acid were added. The mixture was heated for 15 min at 100℃, cooled and absorbance at 532 nm measured. One unit of antioxidant activity was defined as the amount of antioxidant which leads to the same value of ΔOD_{532} (OD_{532} (control)—OD_{532} (sample)) as 1 μmol of mannitol does.

Purification of Antioxidants in PLx —— Methanol precipitation: PLx (pH 3.8)(120 ml) was mixed with methanol (80 ml), and then centrifuged at 10000 × *g* for 10 min at 4℃. The resulting supernatant was lyophilized, and dissolved in 10 ml of distilled water.

Sephadex G-50 gel chromatography: The fraction from the previous step (10 ml) was subjected to gel filtration on a Sephadex G-50 column (33 × 850 mm, 727 ml) preequibrated with distilled water, and eluted with distilled water at a flow rate of 0.4 ml/min, and 80 drops per fraction were collected. The collected active fractions (zone B or zone C) were lyophilized, and dissolved in 10 ml of distilled water.

Separation by reverse-phase-HPLC (RP-HPLC): RP-HPLC was performed on a DELTA 3000 (Waters, U.S.A.) equipped with an UV detector operated at 210 nm. The samples (50 μl for zone B, 50 μl for zone C, see Fig. 2) filtrated with Ekicrodisc® 25(Gelman Sciences Japan, Ltd.) were applied to an ODS Mightysil RP-18 GP column (5 μm-pore size, 10 × 250 mm, 19.6 ml, Cica Merck, Japan) and eluted with an acetonitrile/water linear gradient system (0—40% acetonitrile for 60 min) at a flow rate of 1.0 ml/min.

Identification of Antioxidants Purified from PLx —— NMR spectrometry: The ^{1}H- and ^{13}C-NMR spectra were acquired at 270 MHz and 500 MHz using JEOL JMS GX-270 and JEOL JNM-LA 500 (Tokyo, Japan), respectively, in D_2O or methanol-d_4. Chemical shifts are reported relative to the solvent or relative to tetramethylsilane (TMS) internal standard and coupling constants are reported in Hz. The following abbreviations are used: d; doublet, dd; double-doublet, m; multiplet.

Fast Atom Bombardment Mass (FAB-MASS) spectrometry: The measurements were performed on a JEOL JMS SX-102A. The FAB-MASS spectra were recorded on B/E linked scan under a constant ion accelerating voltage (10 kV). Glycerol was used as a matrix of positive mode.

UV spectrometry: A Hitachi U-2000 spectrophotometer was used in this analysis. Samples were dissolved in distilled water, and their pH showed approximately 6.5.

Retention time by HPLC: HPLC retention times were obtained by the method described above.

RESULTS

Antioxidant Activity of PLx

Using the conjugated dienes method (Fig. 1A), the increasing rate of absorbance per minute

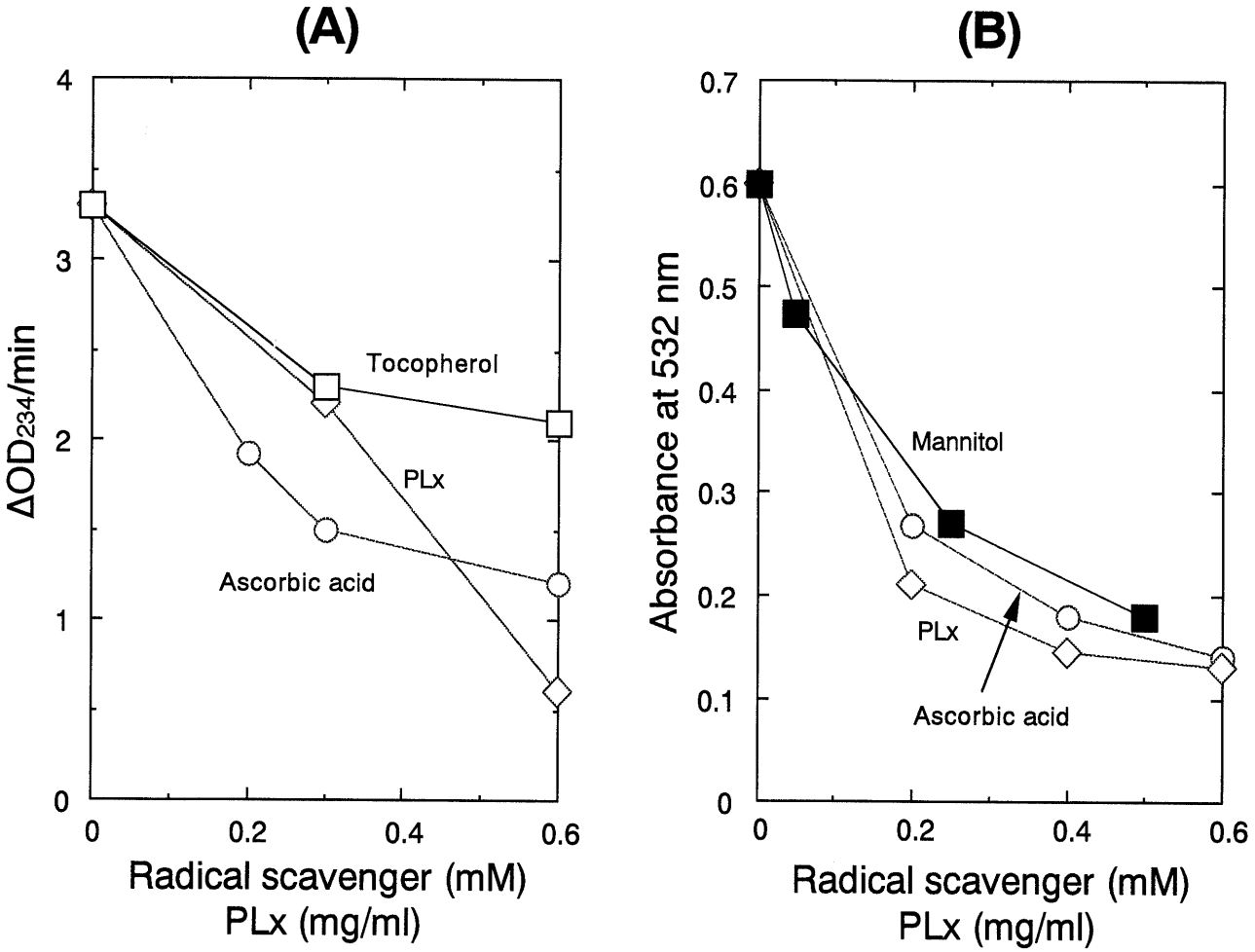

Fig. 1. Antioxidant Activity of PLx

(A) Antioxidant activity of PLx and radical scavengers on conjugated diene production by Fenton Reaction. The antioxidant activity at the indicated final concentration was plotted as the rate of change at OD_{234}/min. Rates were measured from 0 to 1 min following the addition of ferrous ammonium sulfate as described in "Materials and Methods." (B) Antioxidant activity of PLx and radical scavengers on MDA production by Fenton Reaction. MDA production was monitored at 532 nm as described in "Materials and Methods." Each value represents the mean of triplicate determination.

at 234 nm (ΔOD_{234}/min) was abolished by PLx in a dose dependent manner. This result indicated that PLx prevented hydroxyl radicals from generating conjugated dienes of linoleic acid. By the deoxyribose method (Fig. 1B), malondialdehyde (MDA) formation was inhibited by PLx, as observed by mannitol and ascorbic acid. These results showed that PLx possessed antioxidant activity.

Purification of Antioxidants in PLx

A preliminary examination using gel chromatography (data not shown) indicated that the main antioxidant activity in PLx did not reside predominantly in large molecular weight substances (2000 daltons) such as peptides and proteins. On this basis, the first stage was to remove protein as much as possible by the 40% methanol precipitation procedure. PLx (7220 units) was mixed with methanol. Approximately 85% (6140 units) of antioxidant activity measured by the deoxyribose method remained in the supernatant of PLx treated with 40% methanol. This supernatant was applied to Sephadex G-50 gel column chromatography. The elution patterns of antioxidant activity and absorbance at 280 nm are shown in Fig. 2. Maximum antioxidant activity was seen in fraction 73. Fractions having antioxidant activity were divided into two zones, zone B and C (Fig. 2). The combined recovery of antioxidant activity of zone B (fraction 56—72) and C (fraction 73—85) was approximately 70% (5220 units). Fractions from zones B and C were lyophilized and dissolved in 10 ml of distilled water (Fig. 2), and then applied to RP-HPLC.

Fig. 3A and Fig. 3B shows the UV absorption at 210 nm and antioxidant activity after HPLC separation of zone C, respectively. The antiox-

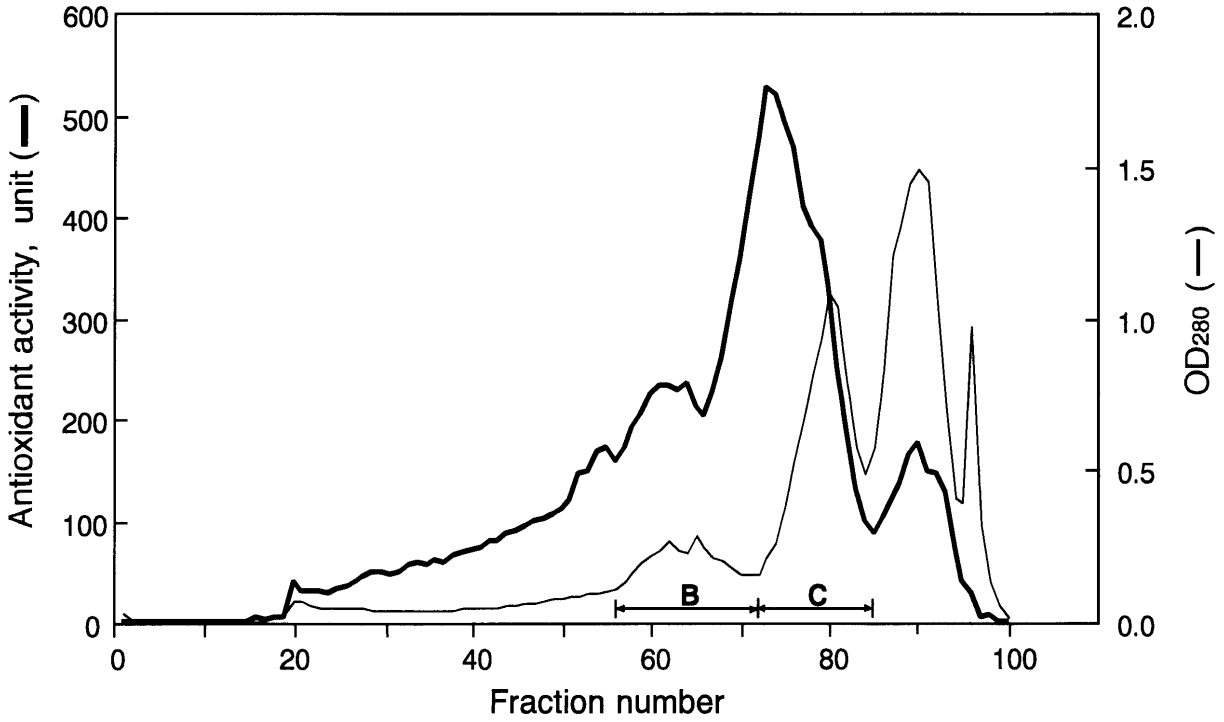

Fig. 2. Sephadex G-50 Chromatography of PLx Extract after 40% MeOH Treatment

Supernatant of PLx treated with 40% MeOH was subjected to Sephadex G-50 chromatography: column size; 850×i.d. 33 mm (727 cm³), eluent; H_2O, flow rate; one drop/15 sec; sample volume; 10 ml, and 80 drops/fraction. UV absorbance (280 nm)(——) of each fraction was measured by Hitachi U-2000 spectrometer. Zone B was composed of fractions from No. 56 to No. 72, and zone C was from No. 73 to No. 85. Antioxidant activity (——) is expressed as μmol equivalents of mannitol by the deoxyribose method.

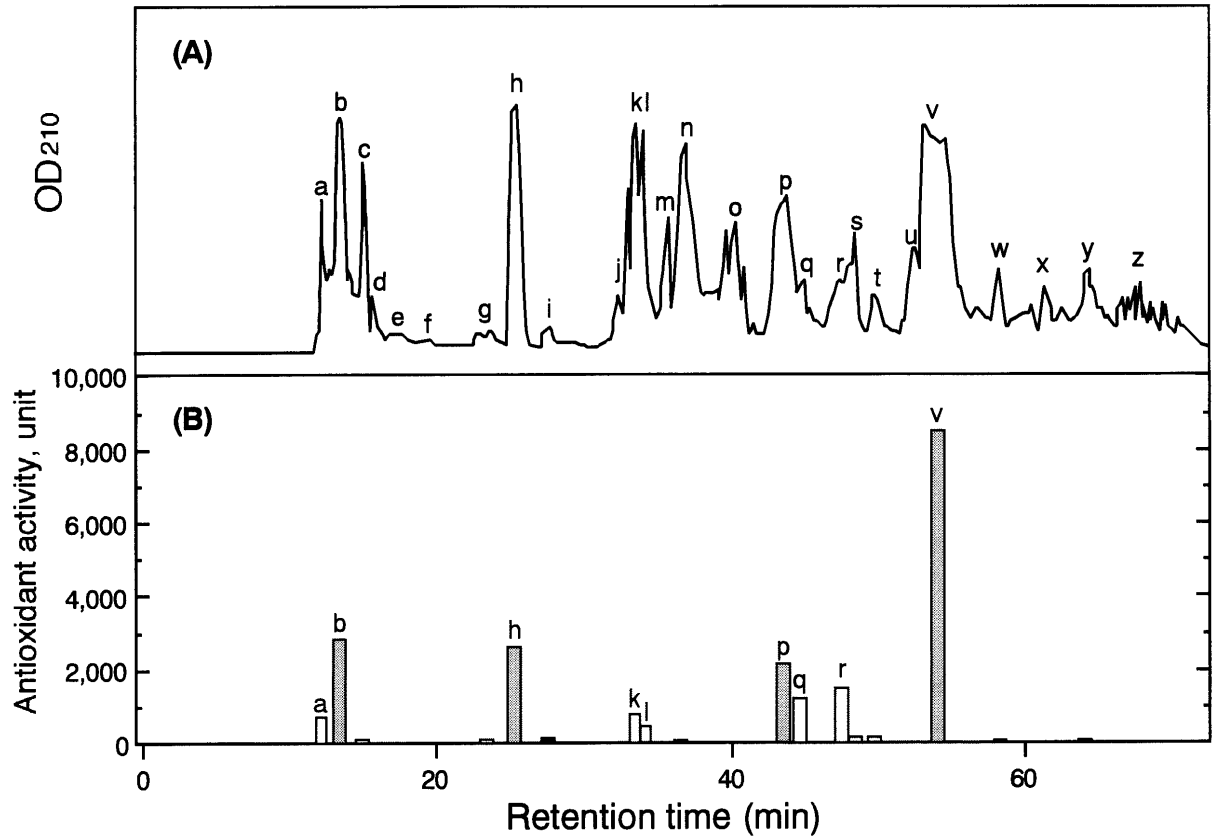

Fig. 3. Separation of Antioxidants in PLx by HPLC (A) and Antioxidant Activity of the Peaks (B)

Sample derived from zone C fraction in Fig. 2 was subjected to RP-HPLC: column; ODS Mightysil RP-18 GP (5 μm), column size; 250×i.d. 10 mm (19.6 cm³), eluent; linear gradient of acetonitrile in H_2O (0—40% (v/v)) containing 0.1% (v/v) trifluoroacetic acid, Flow rate; 1.0 ml/min for 60 minutes, sample volume; 50μl. UV absorbance (210 nm) of each fraction was measured by the detector. Antioxidant activity is expressed as μmol equivalents of mannitol by the deoxyribose method.

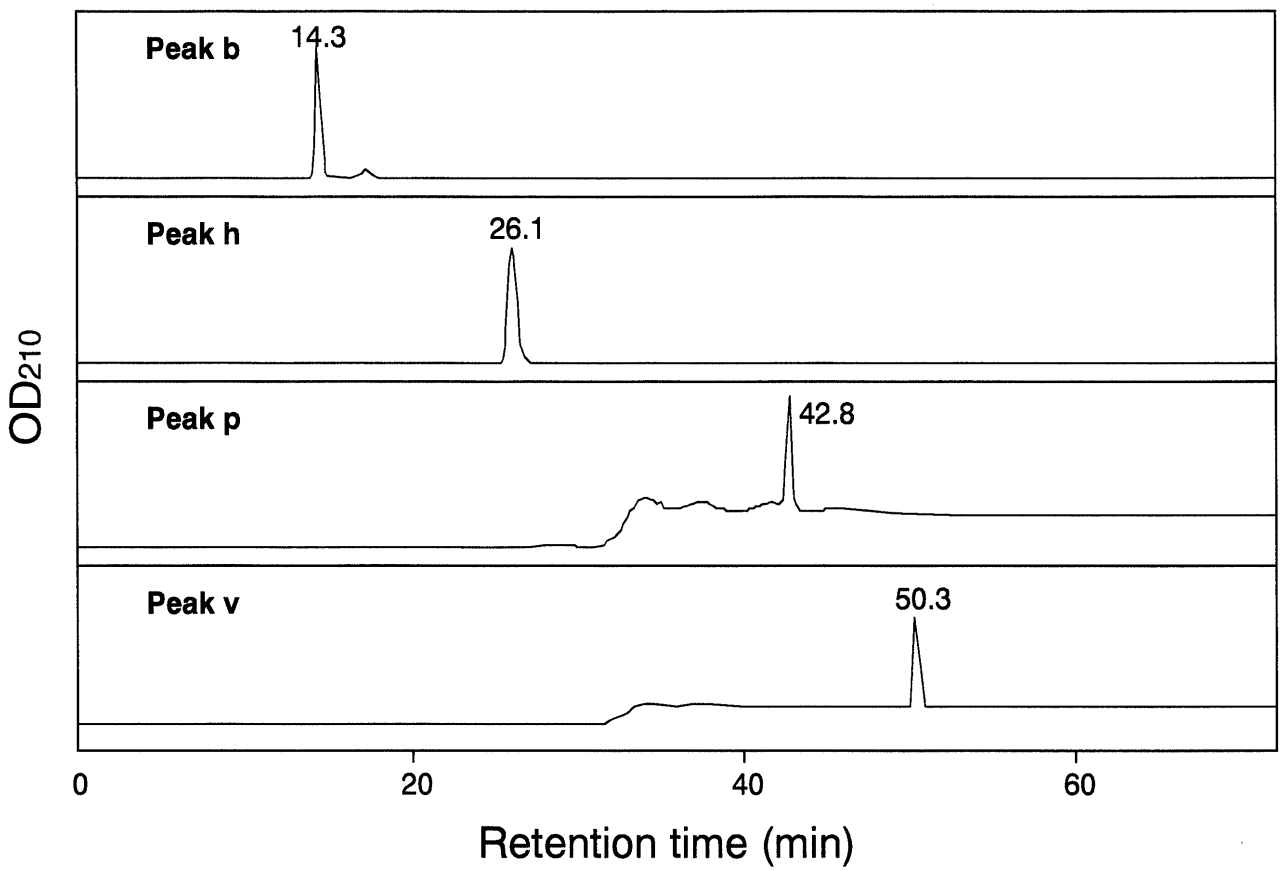

Fig. 4. Rechromatography of Antioxidants (Peaks b, h, p, and v) Separated by HPLC

Peaks b, h, p, and v obtained from Fig. 3 were rechromatographed by HPLC under the same conditions as described in Fig. 3.

idant activity of each peak fraction (peaks a—z, Fig. 3A) is shown in Fig. 3B. Peaks b, h, p, and v showed high antioxidant activity. The retention times of peaks b, h, p, and v were 13.7, 25.2, 43.7, and 53.5 min, respectively. These results suggested that the antioxidants in peaks b, h, p, and v represented a majority of the antioxidants in PLx. The recovery of antioxidant activity from all peaks was approximately 90% (3650 units) of all antioxidant activities applied to the HPLC. Total antioxidant activities of peaks h, p, and v accounted for about 82% (3290 units) of all antioxidant activities applied to the HPLC. The antioxidant activity in peak v represented the largest percentage of total antioxidant activity in PLx (Fig. 3B). Zone B showed a similar elution pattern to zone C, except for the absence of peak h (data not shown).

The purity of each antioxidant (peaks b, h, p, and v) was examined by rechromatography (Fig. 4). Except for peak b, rechromatographed peaks h, p, and v were symmetrical, sharp, and single. The rechromatographed peak b resulted in two peaks as shown in Fig. 4. The minor peak, eluted at a retention time of 17.3 min, accounted for 10% of the total area based on UV absorption. For peak b, we used the rechromatographed peak eluting at a retention time of 14.3 min for identification.

Identification of Antioxidants in PLx

^{1}H-NMR and ^{13}C-NMR spectra of peak h indicated that the compound was uracil, by comparison with an authentic uracil standard. As shown in Tables 1 and 2, ^{1}H-NMR and ^{13}C-NMR chemical shifts of the authentic uracil standard and the compound in peak h were identical. In Table 2, the ^{13}C-NMR chemical shift of peak h corresponding to δ: 152.10 ppm of the uracil standard did not appear. However, the signal intensity at δ: 152.10 ppm for the uracil standard was extremely weak, so that the signal was hard to distinguish from noise. Results of NMR analysis indicated that the compound in peak h may be uracil.

Fig. 5 shows FAB-MASS spectra on the linked scan of authentic uracil standard and peak h. The uracil spectra (peaks 70, 96, 113 m/z) were

Table 1. ^{1}H-NMR Chemical Shifts of Uracil and Peak h (ppm) (Coupling Constants in Parenthesis)

Uracil	peak h
7.53	7.53
(2H, d, J=8 Hz)	(2H, d, J=8 Hz)
5.80	5.80
(2H, d, J=8 Hz)	(2H, d, J=8 Hz)

Table 2. ^{13}C-NMR Chemical Shifts of Uracil and Peak h (ppm)

Uracil	peak h
168.30	168.27
153.23[a)]	Not detected
144.25	144.22
101.87	101.86

a) The intensity of the uracil signal was very weak.

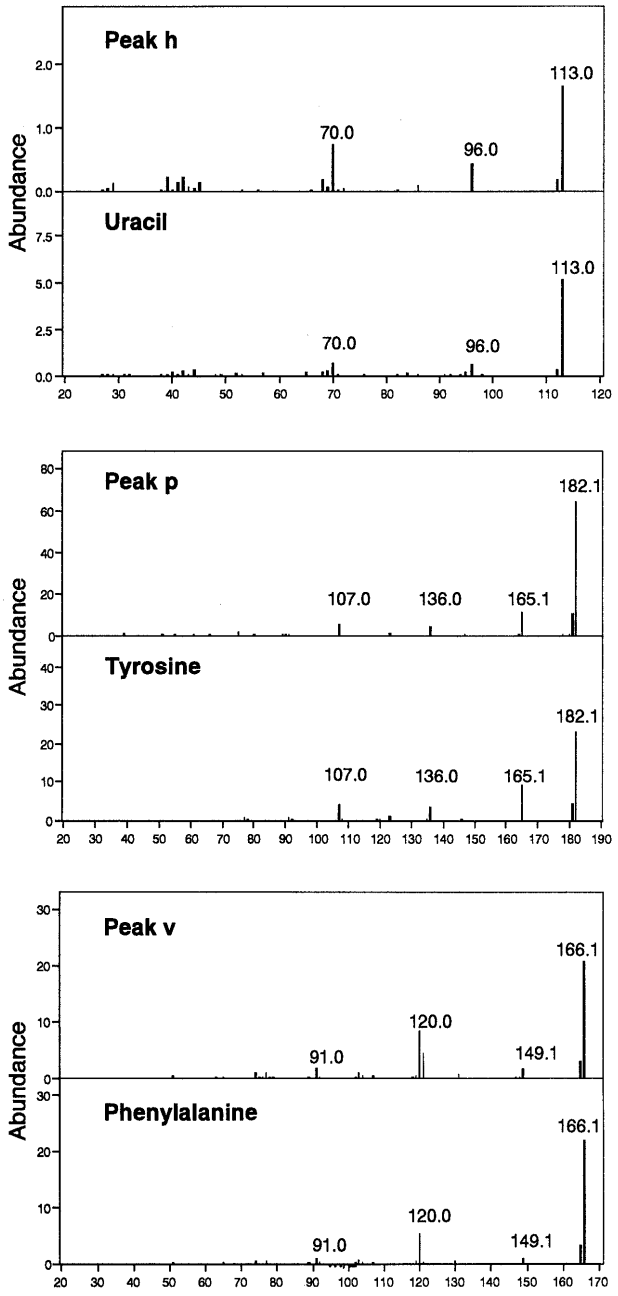

Fig. 5. FAB-MASS Spectra on Linked Scan of Samples

The FAB-MASS spectra of peaks h, p, and v purified by rechromatography were recorded on B/E linked scan on JEOL JMS SX-102A under a constant ion accelerating voltage (10 kV) as described in "Materials and Methods." Glycerol was used as a positive mode matrix.

similar to those of peak h. The UV spectra of peak h and the authentic uracil standard were also similar (Fig. 6). The HPLC retention time of the uracil standard corresponded to that of peak h (data not shown). Specific antioxidant activity (units/mol) of uracil is much higher than the well-known antioxidant, mannitol (Fig. 7). These results (NMR, FAB-MASS, UV, HPLC retention time, and antioxidative activity by the deoxyribose method) revealed that peak h was uracil.

Peak p was examined by the same procedures as described above. NMR data for peak p suggested that it may be tyrosine. ^{1}H-NMR and ^{13}C-NMR chemical shifts of the authentic tyrosine standard and the compound in peak p (Tables 3 and 4) correspond very well. FAB-MASS spectra (Fig. 5), UV spectra (Fig. 6) and HPLC of peak p were identical with those of the authentic tyrosine standard (data not shown), suggesting that peak p is tyrosine. In the ^{13}C-NMR spectrum, tyrosine has 7 different carbon atoms and 7 signals instead of 6 signals should appear in Table 4. However, one signal for both the tyrosine standard and peak p was lacking probably because it was coincident with another peak or its intensity was very weak.

NMR information for peak v in Tables 5 and 6 revealed that it may be phenylalanine. FAB-MASS spectra (Fig. 5), UV spectra (Fig. 6), and HPLC retention time (data not shown) indicated that peak v is consistent with phenylalanine.

In addition, tyrosine and phenylalanine possessed antioxidant activity to approximately the same extent, and both amino acids were more potent antioxidants than mannitol, and the highest specific antioxidant activity was seen in uracil (Fig. 7).

DISCUSSION

In this study, we isolated and identified

Table 3. ^{1}H-NMR Chemical Shifts of Tyrosine and Peak p (ppm) (Coupling Constants in Parenthesis)

Tyrosine	Peak p
7.18	7.14
(2H, d, J=9 Hz)	(2H, d, J=9 Hz)
6.85	6.82
(2H, d, J=9 Hz)	(2H, d, J=9 Hz)
3.85	4.08
(1H. dd, J=8 and 5 Hz)	(1H. dd, J=8 and 6 Hz)
3.22	3.21
(1H. dd, J=15 and 5 Hz)	(1H. dd, J=14 and 6 Hz)
3.00	3.00
(1H. dd, J=15 and 8 Hz)	(1H. dd, J=14 and 8 Hz)

Table 4. ^{13}C-NMR Chemical Shifts of Tyrosine and Peak p (ppm)

Tyrosine	Peak p
157.51	157.21
132.43	131.78
128.61	126.82
117.52	116.93
58.23	55.93
37.49	36.94

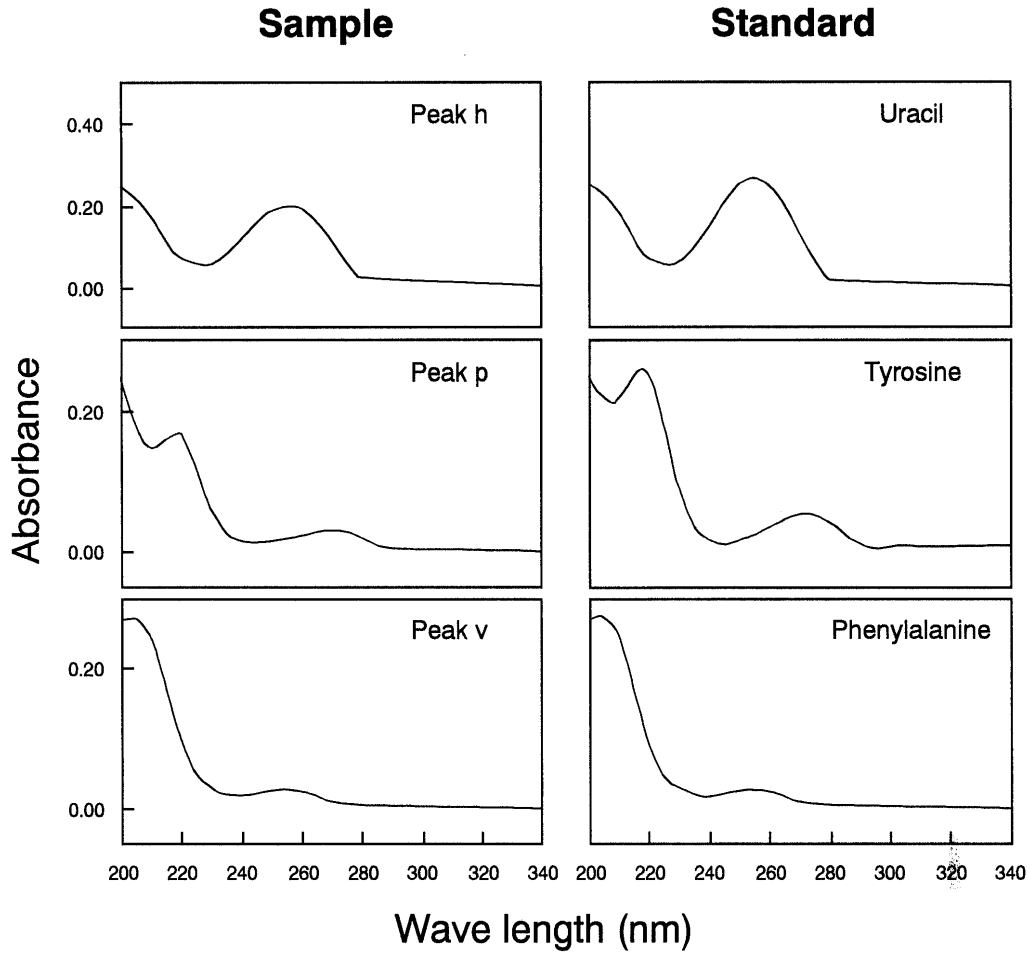

Fig. 6. UV Spectra of Purified Antioxidants (Peaks h, p, and v)

Spectra of samples (rechromatographed peaks h, p, and v) and standards (uracil, tyrosine, and phenylalanine) dissolved in H_2O were measured by Hitachi U-2000 spectrometer.

uracil, tyrosine, and phenylalanine as the main antioxidants in human-PLx, as determined by the deoxyribose method, HPLC retention time, UV spectra, ^{1}H- and ^{13}C-NMR, and FAB-MASS.

Recently, Takehara *et al.* reported that in the late gestational stage, placental tissue suppresses lipid peroxide formation, and that at this stage, the concentration of lipid peroxides in umbilical cord blood was low, and the fetus was protected from reactive oxygens and free radical-induced toxicity by stimulating the activities of some important antioxidant factors in placental tissue.[18] In the current study, these important antioxidant factors were found to include uracil,

Table 5. ^{1}H-NMR Chemical Shifts of Phenylalanine and Peak v (ppm) (Coupling Constants in Parenthesis)

Phenylalaine	Peak v
7.31—7.43	7.02~7.14
(5H, m)	(5H, m)
3.99	3.88
(1H, dd, J=8 and 5 Hz)	(1H, dd, J=8 and 5 Hz)
3.29	3.03
(2H, dd, J=15 and 5 Hz)	(2H, dd, J=14 and 5 Hz)
3.12	2.88
(2H, dd, J=15 and 8 Hz)	(2H, dd, J=14 and 8 Hz)

Table 6. ^{13}C-NMR Chemical Shifts of Phenylalanine and Peak v (ppm)

Phenylalanine	Peak v
173.72	171.40
137.33	135.68
130.41	130.47
129.97	130.12
128.39	128.84
57.60	55.26
38.41	37.38

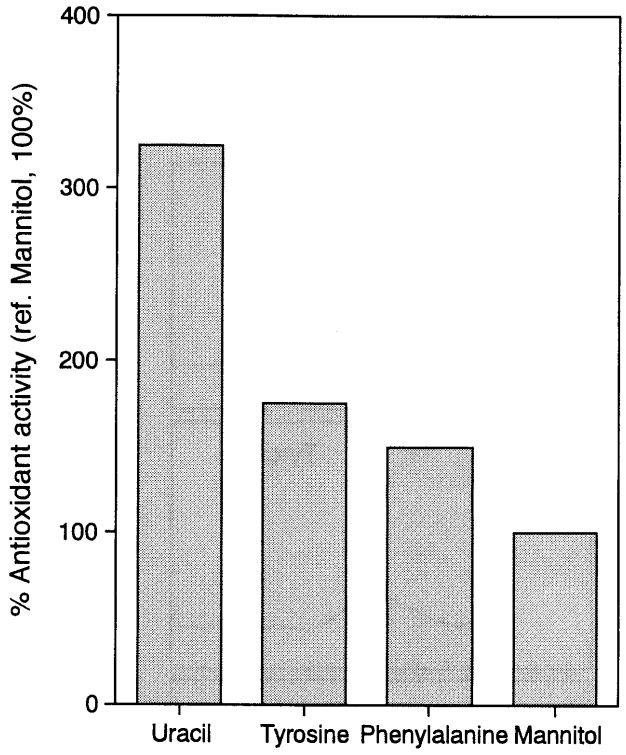

Fig. 7 Comparison of Antioxidant Activity of Uracil, Tyrosine, Phenylalanine and Mannitol

Antioxidant activity was measured by the deoxyribose method using uracil, tyrosine, phenylalanine and mannitol at a concentration of 30μM.

tyrosine, and phenylalanine, while there are still some other unidentified antioxidants in PLx. Based on the HPLC profile, the concentration of uracil, tyrosine, and phenylalanine in the 10% crude PLx was estimated to be 1.3 mM, 4.1 mM, and 11 mM respectively. The percentage antioxidant activity of the three compounds was approximately 46% of the total antioxidant activity.

Previously, the mechanism of antioxidant activity of tyrosine and phenylalanine has been studied.[19,20] In the case of phenylalanine, hydroxyl radicals attack the hydrogen atom located at the para position of the phenylalanine benzyl group (C4), and are converted into H_2O. At the same time, phenylalanine forms a phenylalanine radical, which combines with other hydroxyl radicals and is converted to tyrosine. As a result, when phenylalanine is exposed *in vivo* to hydroxyl radicals, tyrosine is produced as a final product. It is possible that the tyrosine present in PLx is a byproduct of phenylalanine oxidation. On the other hand, in the presence of hydroxyl radicals but not O_2 and $O_2\cdot^-$, tyrosine is transformed to tyrosine radical as an intermediate compound, whereby hydroxyl radicals remove hydrogen atoms at the phenyl meta position (C3 or C5). Hydroxyl radicals then bind with other hydroxyl radicals and bityrosines are created. Tyrosine and phenylalanine probably act as antioxidants *via* these mechanisms when PLx is administered *in vivo*.

Among recent studies on amino acids as antioxidants, Gelvan *et al.* indicated that phenylalanine was a strong Cu(II) chelator and, through its metal (Cu(II))-binding properties, phenylalanine displayed antioxidant activity.[21] Basically, chelating agents reveal antioxidant activity by suppressing the generation of free transition metals (*e.g.* Fe^{2+}, Cu^{2+}). On the other hand, some literature suggests that copper ions may facilitate peroxide decomposition under certain circumstances.[22-24] Further studies are needed to clarify the role of phenylalanine and copper ions in *in vivo* antioxidant activity.

Kapiotis *et al.* suggested that tyrosine is an efficient physiological antioxidant which inhibits LDL oxidation and endothelial cell cytotoxicity initiated by superoxide and nitric oxide radicals.[19] Our study showed that by the deoxyribose method, tyrosine and phenylalanine possessed almost the same specific antioxidant ability (Fig. 7). It is possible that phenylalanine is as effective

as tyrosine in the role of physiological antioxidant.

Uracil as an antioxidant has not been studied substantially compared with uracil derivatives (propylthiouracil, methyluracil *etc.*). Further studies are required to clarify the mechanism of uracil as an antioxidant and its role in human health.

In conclusion, our data indicate that uracil, tyrosine, and phenylalanine are the major antioxidant substances in human PLx.

Acknowledgments This investigation was supported in part by the Ministry of Education, Science, Sports, and Culture of Japan.

REFERENCES

1) Halliwell B., *FASEB J.*, **1**, 358—364 (1987).
2) Tribble D.L., Aw T.Y., Jones D.P., *Hepatology*, **7**, 377—386 (1987).
3) Akkus I., Kalak S., Vural H., Caglayan O., Menekse E., Can G., Durmus B., *Clin. Chim. Acta*, **244**, 221—227 (1996).
4) Biesalski H.K., Frank J., *Immun. Infekt*, **23**, 166—173 (1995).
5) Duthie G.G., Beattie J.A., Arthur J.R., Franklin M., Morrice P.C., James W.P., *Nutrition*, **10**, 313—316 (1994).
6) Niwa Y., *Fragrance J.*, **16**, 30—40 (1988).
7) Odagiri M., Uchiyama S., Imai T., Imamura J., *Oyo Yakuri*, **33**, 621—629 (1987).
8) Itoh H., *Fragrance J.*, **6**, 67—71 (1990).
9) Itoh H., Matsuda R., Nishizaki I., *J. Soc. Comet. Chem. Japan.*, **23**, 267—271 (1990).
10) Itoh H., Matsuda R., Nishizaki I., *J. Soc. Comet. Chem. Japan.*, **22**, 109—117 (1988).
11) Hatae S., Mishima Y., *Fragrance J.*, **6**, 105—108 (1990).
12) Hayakawa R., Matsunaga K., Suzuki M., Hosokawa K., *Skin Res.*, **30**, 663—668 (1988).
13) Ishii M., Hamanaka T., Kimura F., Tsuruda K., Nagahama Y., Saijo T., Yamashita S., Kawahara K., Koyama, E., Yoshimura A., *J. Med. Soc. Toho*, **18**, 32—66 (1971).
14) Sakurane K., Horiguti Y., Sugai T., Inui H., *Dermatol. Urol.*, **25**, 388—392 (1963).
15) Takanashi K., Watanabe K., Yoshizawa I., *Biol. Pharm. Bull.*, **16**, 217—219 (1993).
16) Kharasch E.D., Novak R.F., *J. Biol. Chem.*, **260**, 10645—10652 (1985).
17) Rice-Evans C.A., Diplock A.T., Symons M.C.R., "Techniques in Free Radical Research," vol. 22, ed. by Burabon R.H., and van Knippenberg P.H., Elsevier, Amsterdam, pp. 41—44 (1991).
18) Takehara Y., Yoshioka T., Sasaki J., *Acta Med. Okayama*, **44**, 103—111 (1990).
19) Kapiotis S., Hermann M., Held I., Mühl A., Gmeiner B., *FEBS Lett.*, **409**, 223—226 (1997).
20) Aeschbach R., Amado R., Neukom H., *Biochim. Biophys. Acta*, **439**, 292—301 (1976).
21) Gelvan D., Moreno V., Gassmann W., Hegenauer J., Saltman P., *Biochim. Biophys. Acta*, **1116**, 183—191 (1992).
22) Beckman J.K., Borowitz S.M., Greene H.L., Burr I.M., *Lipids*, **23**, 559—563 (1988).
23) Gutteridge J.M.C., *Biochem. Biophys. Res. Commun.*, **77**, 379—386 (1977).
24) Gutteridge J.M.C., Xiao-Chang F., *Biochem. Biophys. Res. Commun.*, **99**, 1354—1360 (1981).