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# Pectin Hydroxamic Acids Exhibit Antioxidant Activities in Vitro

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Commercial pectins with different degrees of esterification (DE) were reacted with equal volumes of 2 M alkaline hydroxylamine (pH 12.0) at room temperature for 4 h to prepare pectin hydroxamic acids (PHAs; DE94T4, DE65T4, and DE25T4) according to a previously reported method (Hou et al., *J. Agric. Food Chem.* **2003**, *51*, 6362−6366) and were used to test the antioxidant and antiradical activities in comparison with those of DE94, DE65, and DE25 pectins. The half-inhibition concentrations, $IC_{50}$, of scavenging activity against DPPH were 1.51, 5.43, and 5.63 mg/mL for DE94T4, DE65T4, and DE25T4, respectively, and were much lower than those of corresponding DE pectins under the same concentrations. The scavenging activities of PHAs for DPPH radicals were positively correlated with original DE values of pectin. The optimal pH of DE94T4 for scavenging DPPH radicals was 7.9 or 8.0. Using electron spin resonance (ESR) for scavenging hydroxyl radicals, under the same concentrations of 125 $\mu$g/mL, DE94T4, DE65T4, and DE25T4, respectively, exhibited 73.53, 69.01, and 55.17% antiradical activities. PHAs also exhibited protection against hydroxyl radical-mediated DNA damage and anti-human low-density lipoprotein peroxidation tests.

**KEYWORDS: Antioxidant activity; DPPH radicals; electron spin resonance (ESR); low-density lipoprotein (LDL); pectin hydroxamic acids (PHAs)**

## INTRODUCTION

Active oxygen species (or reactive oxygen species) and free radical-mediated reactions are involved in degenerative or pathological processes such as aging (*1*, *2*), cancer, coronary heart disease, and Alzheimer's disease (*3*−*6*). There have been several reports concerning natural compounds in fruits and vegetables for their antioxidant activities, such as anthocyanin (*7*), water extracts of roasted *Cassia tora* (*8*), and the storage proteins of of sweetpotato root (*9*), yam tuber (*10*), yam mucilages (*11*), and potato tuber (*12*).

A variety of hydroxamic acid derivatives have been reported to have pharmacological and biological activities toward cancer, cardiovascular diseases, Alzheimer's disease, tuberculosis, etc (*13*). Succinimide hydroxamic acids were provn to be potent inhibitors of histone deacetylase and tumor cell proliferation (*14*). Hydroxamic acid derivatives of salicylic acid were cyclooxygenase (COX)-1 and COX-2 inhibitors (*15*). Oxal hydroxamic acid derivatives were potent inhibitors of matrix metalloproteinases (*16*). The aspartic acid $\beta$-hydroxamate exhibited antitumor activity on L5178Y leukemia (*17*), therapeutic effect on friend erythroleukemia (*18*), and antiproliferative activity on friend virus-infected erythropoietic progenitor cells (*19*). We have also reported that the different degrees of esterification (DE) of pectin hydroxamic acids (PHAs) exhibited both semicarbazide-sensitive amine oxidase and angiotensin converting enzyme (ACE) inhibitory activities (*20*). In this paper, these PHAs were used to investigate the antioxidant and antiradical activities in comparison with those of DE94, DE65, and DE25 pectins. The results showed that PHAs exhibited antioxidant and antiradical activities.

## MATERIALS AND METHODS

**Materials.** 2,2-Diphenyl-1-picrylhydrazyl (DPPH), 5,5-dimethyl-1-pyrroline-*N*-oxide (DMPO), ferrous sulfate, and human low-density lipoprotein (LDL) were purchased from Sigma Chemical Co. (St. Louis, MO). Hydrogen peroxide (33%) was from Wako Pure Chemical Industry (Osaka, Japan). Calf thymus DNA (activated, 25 $A_{260}$ units $mL^{-1}$) was purchased from Amersham Biosciences (Uppsala, Sweden). Other chemicals and reagents were from Sigma Chemical Co.

**Scavenging Activities of DPPH Radicals by Spectrophotometry.** Three tenths of a milliliter of DE94T4, DE94, DE65T4, DE65, DE25T4, and DE25 (final concentrations, 0.5, 1, 2, 4, and 6 mg/mL) was added to 0.1 mL of 1 M Tris-HCl buffer (pH 7.9) and then mixed with 0.6 mL of 100 $\mu$M DPPH in methanol to the final concentration of 60 $\mu$M for 20 min under light protection at room temperature (*11*, *12*). The decrease of absorbance at 517 nm was measured and expressed as $\Delta A_{517nm}$. Deionized water was used as a blank experiment. Means of triplicates were measured. The scavenging activity of DPPH radicals (percent) was calculated with the equation $(\Delta A_{517,blank} - \Delta A_{517,sample}) \div \Delta A_{517,blank} \times 100\%$. $IC_{50}$ is the concentration of half-inhibition.

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**Optimal pH for Scavenging DPPH Radicals.** DE94T4 (1 mg/mL) was used to investigate the optimal pH for DPPH scavenging activities. The Tris-HCl buffer of different pH values (7.0−8.5) was used for comparisons. The scavenging activity of DPPH radicals (percent) was calculated with the equation $(\Delta A_{517,blank} - \Delta A_{517,sample}) \div \Delta A_{517,blank} \times 100\%$.

**Scavenging Activity of Hydroxyl Radicals by ESR Spectrometry.** The hydroxyl radical was generated by Fenton reaction according to the method of Kohno et al. (*21*). The total 500 $\mu$L mixture included 125 $\mu$g/mL DE94T4, DE65T4, and DE25T4, 5 mM DMPO, and 0.05 mM ferrous sulfate. After mixing, the solution was transferred to an ESR quartz cell and placed at the cavity of the ESR spectrometer, and then hydrogen peroxide was added to a final concentration of 0.25 mM. Deionized water was used instead of sample solution for blank experiments. After 40 s, the relative intensity of the signal of theDMPO−OH spin adduct was measured. All ESR spectra were recorded at the ambient temperature (298 K) on a Bruker EMX-6/1 ESR spectrometer equipped with WIN-ESR SimFonia software version 1.2. The conditions of ESR spectrometry were as follows: center field, 345.4 ± 5.0 mT; microwave power, 8 mW (9.416 GHz); modulation amplitude, 5 G; modulation frequency, 100 kHz; time constant, 0.6 s; scan time, 1.5 min.

**Protection against Hydroxyl Radical-Induced Calf Thymus DNA Damage by PHAs.** The hydroxyl radical was generated by Fenton reaction according to the method of Kohno et al. (*21*). The 45 $\mu$L reaction mixture included DE94T4 and DE25T4 (final concentrations of 0.11, 0.22, 0.55, and 5.6 mg/mL), 15 $\mu$L of calf thymus DNA, 18 mM $FeSO_4$, and 60 mM hydroxygen peroxide at room temperature for 15 min. Ten microliters of 1 mM EDTA was added to stop the reaction. Calf thymus DNA only was used for the blank test, and the control test was without DE94T4 or DE25T4 addition. After the agarose gel electrophoresis, the treated DNA solutions were stained with ethidium bromide and observed under UV light.

**Protection against $Cu^{2+}$-Induced LDL Peroxidation by PHAs.** The capacity of DE94T4, DE65T4, and DE25T4 (final concentrations of 57.2, 66.8, 100, 132, and 400 $\mu$g/mL) against $Cu^{2+}$-induced human LDL oxidation in a total 1.1 mL sample volume was measured by relative mobility on the 0.5% agarose gel after staining by Sudan Black B (*22*). The LDL (0.5 mg of protein/mL) was incubated at 37 °C under air in 10 mM phosphate buffer (pH 7.4) containing 10 $\mu$M $CuSO_4$ for 24 h with or without PHAs. The peroxidation reaction was stopped by 100 $\mu$M EDTA additions. Mobility of native LDL was assigned a value of 100%, and others were calculated on the relative mobility.

## RESULTS AND DISCUSSION

**Scavenging Activity against DPPH Radicals of PHAs.** A variety of hydroxamic acid derivatives have been reported to have pharmacological and biological activities toward cancer, cardiovascular diseases, Alzheimer's disease, tuberculosis, etc (*13*). We reported that PHAs exhibited both semicarbazide-sensitive amine oxidase and ACE inhibitory activities (*20*). Therefore, the PHAs of DE94T4, DE65T4, and DE25T4 were used to test in vitro antioxidant and antiradical activities in comparison with DE94, DE65, and DE25, respectively. **Figure 1** shows the results of scavenging activity of DPPH radicals. The DPPH radicals were widely used in the model system to investigate the scavenging activities of several natural compounds. The colors change from purple to yellow, and its absorbance at wavelength 517 nm decreases as the result of the formation of DPPH−H through donation of hydrogen by antioxidants (*23*). The PHAs of DE94T4, DE65T4, and DE25T4 showed dose-dependent DPPH radical scavenging activities (**Figure 1A**). The $IC_{50}$ values of scavenging activity against DPPH were 1.51, 5.43, and 5.63 mg/mL for DE94T4, DE65T4, and DE25T4, respectively, and were much lower than those of corresponding DE pectins under the same concentrations. From the results of **Figure 1B**, it was also found that the scavenging activities of PHAs for DPPH radicals were positively correlated with corresponding original DE values (DE94, DE65, and DE25) of pectin. Yale (*24*) reported that in aqueous solution the monohydroxamic acids behaved as weak acids. The dissociation of the hydroxamic acid moiety in PHAs might influence the scavenging activity. Therefore, DE94T4 was chosen for DPPH optimal pH scavenging assay (**Figure 1C**). In the pH range of 7.0−8.5, the optimal pH of DE94T4 for scavenging DPPH radicals was 7.9 or 8.0 (**Figure 1C**). This result revealed that PHAs exhibited antiradical activities. The hydroxamic acid moiety in PHAs might contribute its free radical scavenging activities.

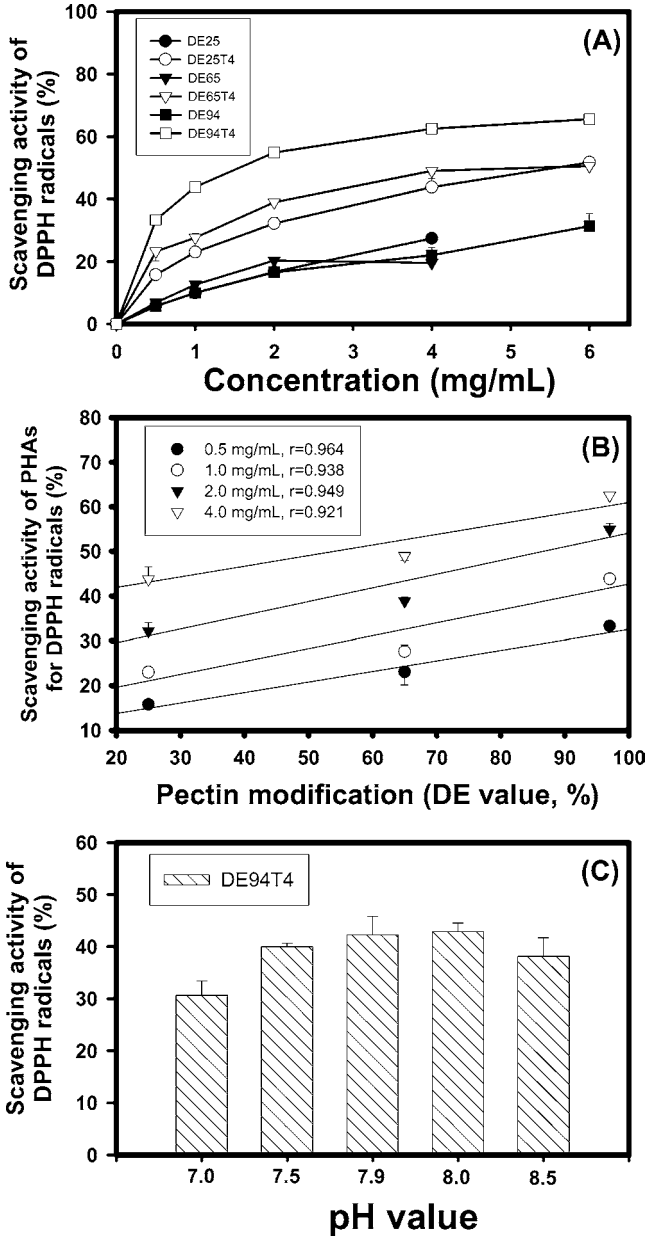


**Figure 1.** (**A**) Effects of different concentrations (0.5, 1.0, 2.0, 4.0, and 6.0 mg/mL) of pectin hydroxamic acid (DE94T4, DE65T4, and DE25T4) and pectin (DE94, DE65, and DE25) on the scavenging activities of DPPH radicals with spectrophotometry; (**B**) correlations between the scavenging activities of PHAs of DPPH radicals and corresponding original DE values (DE94, DE65, and DE25) of pectin under different concentrations (0.5, 1.0, 2.0, and 4.0 mg/mL); (**C**) optimal pH for DPPH scavenging activities of DE94T4 in the ranges of Tris-HCl buffer (pH 7.0–8.5). The scavenging activity of DPPH radicals (percent) was calculated with the equation $(\Delta A_{517,blank} - \Delta A_{517,sample}) \div \Delta A_{517,blank} \times 100\%$.

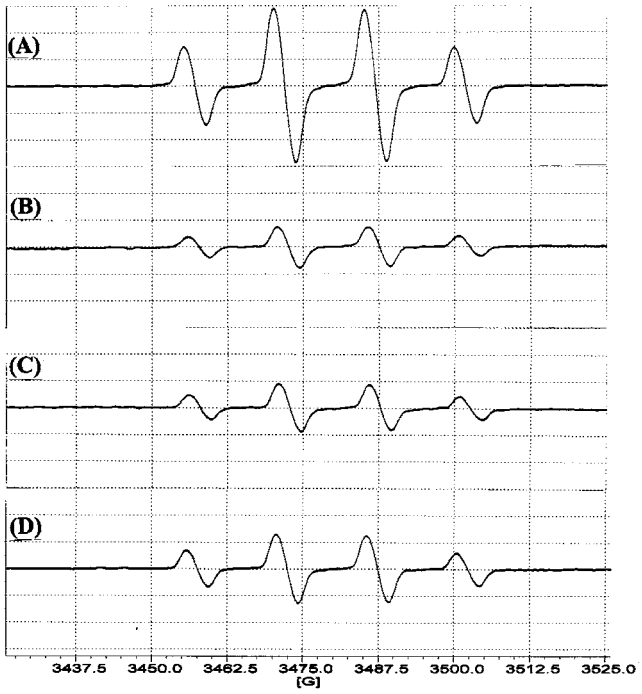


**Figure 2.** Scavenging activity of 125 $\mu$g/mL pectin hydroxamic acids against the hydroxyl radicals measured by ESR spectrometry: (**A**) control; (**B**) DE94T4; (**C**) DE65T4; (**D**) DE25T4. All ESR spectra were recorded at ambient temperature (298 K) on a Bruker EMX-6/1 ESR spectrometer equipped with WIN-ESR SimFonia software version 1.2. Conditions of ESR spectrometry were as follows: center field, 345.4 ± 5.0 mT; microwave power, 8 mW (9.416 GHz); modulation amplitude, 5 G; modulation frequency, 100 kHz; time constant, 0.6 s; scan time, 1.5 min.

**Scavenging Activity of Hydroxyl Radicals by ESR Spectrometry.** The hydroxyl radical was generated by Fenton reaction according to the method of Kohno et al. (*21*) and was trapped by DMPO to form the DMPO−OH adduct. The intensities of the DMPO−OH spin signal in ESR spectrometry were used to evaluate the scavenging activities of DE94T4, DE65T4, and DE25T4 against hydroxyl radicals (**Figure 2**). From the results of DMPO−OH intensities, under the same concentration of 125 $\mu$g/mL, DE94T4, DE65T4, and DE25T4, respectively, exhibited 73.53, 69.01, and 55.17% antihydroxyl radical activities. DE94T4 exhibited a higher scavenging activity than DE65T4 and DE25T4 did. The orders of scavenging hydroxyl radical activities (**Figure 2**) were the same as those of DPPH radicals (**Figure 1**), which meant that the more substituted hydroxamic acid moieties in PHAs, the higher scavenging activities were found. From the results of **Figures 1** and **2**, this is the first report that PHAs of DE94T4, DE65T4, and DE25T4 exhibited antioxidant and antiradical activities.

**Protection against Hydroxyl Radical-Induced Calf Thymus DNA Damage by PHAs.** Free radicals could damage macromolecules in cells, such as DNA, proteins, and lipids in membranes (*25*). **Figure 3** shows the DE94T4 (top) and DE25T4 (bottom) protections against hydroxyl radical-induced calf thymus DNA damage. Calf thymus DNA only was used for the blank test, and the control test was without DE94T4 (top, **Figure 3**) or DE25T4 (bottom, **Figure 3**) addition. Compared to blank test and control test, it was found that the added DE94T4 or DE25T4 above 0.55 mg/mL (lane 4, both panels, **Figure 3**) could protect against hydroxyl radical-induced calf thymus DNA damage after 15 min reactions.

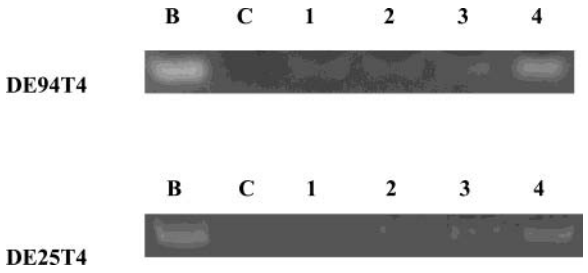


**Figure 3.** Effects of pectin hydroxamic acids (top, DE94T4; bottom, DE25T4) on protection against hydroxyl radical-induced calf thymus DNA damage after 15 min of reaction. After the agarose gel electrophoresis, the treated DNA solutions were stained with ethidium bromide and observed under UV light. Calf thymus DNA only was used for the blank test, and the control test was without DE94T4 or DE25T4 additions. Lanes 1–4 were 0.11, 0.22, 0.55, and 5.6 mg/mL, respectively.

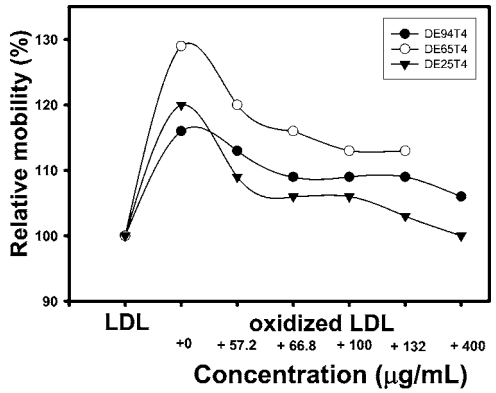


**Figure 4.** Protection against $Cu^{2+}$-induced LDL peroxidation by different concentrations of pectin hydroxamic acids DE25T4 (57.2, 66.8, 100, 132, and 400 $\mu$g/mL). LDL (0.5 mg of protein/mL) was incubated at 37 °C under air in 10 mM phosphate buffer (pH 7.4) containing 10 $\mu$M $CuSO_4$ for 24 h with or without PHAs. EDTA (100 $\mu$M) was added to stop the peroxidation reaction. Effects of protection were measured by relative mobility on the 0.5% agarose gel after staining by Sudan Black B. The mobile distance of native LDL was assumed as 100%.

**Effect of PHAs on Protection against $Cu^{2+}$-Induced Human LDL Peroxidation by Relative Mobility on Agarose Gels.** LDL peroxidation has been reported to contribute to atherosclerosis development (*26*). Therefore, delay or prevention of LDL peroxidation is an important function of antioxidants. The derivatization of lysine residues of apolipoprotein B after LDL peroxidation leads to the net increase in the negative charge and thus to a faster anodic mobility (*26*). Therefore, the slower mobility might be the effect against human LDL peroxidation. The mobile distance of native LDL was assumed as 100%. From the results of **Figure 4**, $Cu^{2+}$-induced oxidized LDL had higher relative mobility (116, 129, and 120%) than that of the native LDL (100%). After the PHAs of DE94T4, DE65T4, and DE25T4 additions, the relative mobility was reduced to 106, 113, and 100%, respectively, for 400, 132, and 400 $\mu$g/mL. It was clear that the PHAs exhibited protection effects against human LDL peroxidation.

In conclusion, the pectin hydroxamic acids (DE94T4, DE65T4, and DE25T4) showed antioxidant activities (**Figures 1**−**4**). The higher DE pectin hydroxamic acid derivatives seemed to exhibit more potent antioxidant or antiradical activities (**Figures 1** and **2**). The resonance properties of hydroxamic acid moieties (R−CONHOH) in the galacturonic acid monomer of pectin molecules might explain the differences between pectin hydroxamic acids and corresponding DE pectins. A variety of hydroxamic

acid derivatives have been reported to have pharmacological and biological activities toward cancer, cardiovascular diseases, Alzheimer's disease, tuberculosis, etc. (*19*). Our previous study also confirmed that PHAs exhibited both semicarbazide-sensitive amine oxidase and angiotensin converting enzyme inhibitory activities (*20*). Except for the food industry of jams and jellies (*27*, *28*), biological activities of PHAs derserve further ivestigations.

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