Enzyme Inhibition and Protein-Binding Action of the Procyanidin-Rich French Maritime Pine Bark Extract, Pycnogenol: Effect on Xanthine Oxidase

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Pycnogenol, an extract from French maritime pine bark (PBE), is a complex mixture of bioflavonoids with reported protective effects against disease. PBE is an effective scavenger of reactive oxygen species, and its main constituents are procyanidins of various chain lengths. To find out the biochemical basis of action of PBE on enzyme activity, involvement of its redox activity and direct binding to the enzyme in its subsequent action on enzyme activity have been investigated. PBE dose-dependently inhibited the activities of xanthine oxidase, xanthine dehydrogenase, horseradish peroxidase, and lipoxygenase, but it did not affect the activities of glucose oxidase, ascorbate oxidase, or elastase. To characterize the mechanism of PBE action, studies were focused on xanthine oxidase and glucose oxidase. Under non-denaturing conditions, PBE changed the electrophoretic mobility of xanthine oxidase but not of glucose oxidase. Gel filtration chromatography confirmed higher molecular weight complexes of xanthine oxidase and xanthine dehydrogenase in the presence of PBE. It was found that hydrophobic bonding might be the dominant mode of interaction between PBE and xanthine oxidase. The importance of the binding in the effect of PBE on enzyme activity was supported by the observation that PBE binds to and inhibits catalase, but not superoxide dismutase. However, no correlation was found between superoxide/hydroxyl radical scavenging activity and the inhibitory effect on xanthine oxidase activity of PBE, various purified flavonoids, or other complex mixtures of bioflavonoids. The results indicate that PBE selectively inhibits xanthine oxidase through binding to the enzyme rather than by the redox activity.

Keywords: *Bioflavonoids; enzyme inhibition; procyanidins; protein binding; Pycnogenol*

INTRODUCTION

Recent interest in polyphenolic compounds has increased greatly due to their ability to scavenge reactive oxygen species as well as the multiple biological activities attributed to these compounds (Bravo, 1998). Therefore, flavonoids alone or as a part of a complex mixture of polyphenols have been used therapeutically in European countries and in the United States as a dietary supplement (Kottke, 1998). Pycnogenol, an extract from French maritime pine bark (PBE), is an example of a highly standardized mixture of certain polyphenolic compounds. The chromatographic profile of PBE after size separation by normal phase high-performance liquid chromatography (HPLC) has been recently reported (Packer et al., 1999). Procyanidins formed by catechin and epicatechin units with a degree of polymerization of up to heptamer are major constituents of PBE and constitute 75% of its weight. PBE also contains monomer flavonoids such as catechin, epicatechin, and taxifolin and phenolcarbonic acids and their glycosides. Recently, various biochemical and pharmacological studies have revealed some interesting properties of PBE, both in vitro and in vivo (Packer et al., 1999). One of the underlying mechanisms for the observed biological effects of PBE could be interaction with cellular proteins, namely, modulation of enzyme activities. However, the biochemical basis of the action of PBE on enzyme activities is not known.

Due to their antioxidant activity, polyphenolic compounds, by donating electrons, could change the redox state of enzymes, particularly those that contain metals as their prosthetic groups or react with free radicals generated at the active site of the enzyme, thereby affecting enzyme activities. Thus, the redox activity of PBE could constitute a possible mechanism for its effect on enzyme activities. It was concluded that the redox activity of monomeric flavonoids is responsible for their effects on enzyme activities (Elliott et al., 1992; Kemal et al., 1987). However, this concept has not been extensively explored concerning procyanidins.

Furthermore, it has been demonstrated that procyanidins bind with high affinity to extended proteins such as proline-rich proteins and histatins (Hagerman and Butler, 1981; Yan and Bennick, 1995). Therefore, one of the mechanisms responsible for the action of PBE on enzymes could be the binding of procyanidins to the enzyme, changing its conformation and thus affecting its activity. In most studies, the inhibitory action of procyanidins on enzyme activity has been attributed to their ability to bind to proteins. Such binding has been indirectly shown by the lack of correlation between enzyme inhibition and the binding of related polyphenols to hemoglobin (Hatano et al., 1990).

Therefore, in the present study, the effect of PBE on the activities of some purified enzymes as well as possible involvement of its redox activity and/or direct

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protein binding, as a model for its interaction with proteins, has been evaluated.

MATERIALS AND METHODS

Chemicals. (±)-Taxifolin, (−)-epicatechin, (−)-epicatechin gallate, (±)-catechin, xanthine, xanthine oxidase from buttermilk, linoleic acid, soybean lipoxygenase, guaiacol, horseradish peroxidase, β-nicotinamide adenine dinucleotide:oxidized form, elastase, Suc-$(Ala)_3$-*p*-nitroanilide, nitroblue tetrazolium, Sepharose 6B, glucose oxidase from *Aspergillus niger*, ascorbic acid, ascorbate oxidase from *Cucurbita* species, riboflavin, superoxide dismutase from bovine erythrocyte, and DMPO were from Sigma Chemical Co. (St. Louis, MO). Catalase from human erythrocyte was from Calbiochem Co. (Loyola, CA). Sephadex G-25 was from Pharmacia, Inc. (Piscataway, NJ). A 3−8% NuPAGE precast gel was from Invitrogen. Other chemicals were obtained from Fischer Scientific (Pittsburgh, PA) or Sigma and were of the highest grade. Procyanidins B1, B2, and C1 from apple and B3 and C2 from barley were a generous gift from Kyowa Hakko Kogyo Co. (Ibaraki, Japan). The purities of the procyanidins were as follows: procyanidins B1, B2, and C1, 98%; procyanidin B3, 94%; and procyanidin B2, 93%. Pycnogenol was obtained from Horphag Research (Geneva, Switzerland). Rat liver xanthine dehydrogenase was a gift from Dr. J. M. McCord of the University of Colorado and was purified as the xanthine oxidase form that can be converted to the dehydrogenase form by incubation with sulfhydryl reducing reagents.

Measurement of Enzyme Activities. *Xanthine Oxidase (XO).* XO activity was determined by following uric acid production at 295 nm ($\epsilon = 11\ mM^{-1}\ cm^{-1}$) in a Shimadzu (Tokyo, Japan) UV160U split-beam UV−visible spectrophotometer (Battelli et al., 1973). The reaction mixture contained 150 μM xanthine and 100 μM EDTA in 0.1 M phosphate buffer, pH 7.4, with or without test compounds. Reactions were started by addition of 12 milliunits/mL XO (9.25−12.6 μg of protein/mL). XO activity was also measured polarographically at 30 °C to confirm the quantitative accuracy of rate measurements made in the presence of taxifolin or Triton X-100.

Lipoxygenase. Lipoxygenase activity was measured by using a Clark oxygen electrode (Yellow Springs Instrument Co., Yellow Springs, OH) with 1 mM linoleic acid in NH_4OH-NH_4Cl buffer, pH 9.0 (Vliegenthart and Veldink, 1982). Reactions were started by addition of 60 units/mL (0.32 μg of protein/mL) of lipoxygenase, and the activity was followed for at least 3 min.

Horseradish Peroxidase (HRP). HRP activity was assayed in a solution containing 3.4 mM guaiacol, 0.9 mM H_2O_2, and 50 mM phosphate buffer, pH 6.0, and the increase in absorbance was followed at 470 nm. The activity was calculated using an extinction coefficient of 26.6 $mM^{-1}\ cm^{-1}$ at 470 nm for tetraguaiacol (Putter, 1975). Reactions were started by addition of 25 milliunits/mL (0.105 μg of protein/mL) of HRP and followed at least for 3 min.

Conversion of the Rat Liver XO to Dehydrogenase Form and Determination of Xanthine Dehydrogenase (XDH) Activity. Rat liver XO was converted to the dehydrogenase form by incubating 150 milliunits of XO with 5 mM DTT in 0.1 M phosphate buffer, pH 7.8, containing 0.4 mM EDTA and 1 mM sodium salicylate at 20 °C for 2 h, followed by passage through a small column of Sephadex G-25 equilibrated with 0.1 M potassium phosphate buffer, pH 7.8, containing 0.4 mM EDTA (Saito and Nishino, 1989). In this manner, >80% of the activity was converted to the dehydrogenase form. Prepared XDH was used immediately in the activity measurement or gel filtration assays. XDH activity was measured spectrophotometrically by following β-NAD^+ reduction at 340 nm ($\epsilon = 6.22\ mM^{-1}\ cm^{-1}$). The reaction mixture contained 150 μM xanthine, 0.1 mM EDTA, and 500 μM β-NAD^+ in 0.1 M phosphate buffer, pH 7.4.

Glucose Oxidase (GO). GO activity was measured polarographically with 10 mM glucose in 0.1 M phosphate buffer, pH 6.5, containing 0.1 mM EDTA at 30 °C (Ilo et al., 1986). Reactions were started by addition of 110 milliunits/mL (0.2 μg of protein/mL) GO.

Ascorbate Oxidase (AO). AO activity was measured polarographically with 1 mM ascorbic acid in 0.1 M Chelex-treated phosphate buffer, pH 7.4, containing 0.1 mM EDTA (Wimalasena and Dharmasena, 1991). Reactions were started by addition of 30 milliunits/mL (0.23 μg of protein/mL) AO. Oxygen consumption due to autoxidation of ascorbate was subtracted from all measurements.

Elastase. Elastase activity was assayed in a solution containing 200 μM Suc-$(Ala)_3$-*p*-nitroanilide and 0.2 M Tris buffer, pH 8.0, and the increase in absorbance was measured at 410 nm. The activity was calculated using an extinction coefficient of 8800 $M^{-1}\ cm^{-1}$ at 410 nm for *p*-nitroaniline (Bieth et al., 1974). Reactions were started by addition of 19 milliunits/mL (3.85 μg of protein/mL) elastase.

Catalase. Catalase activity was assayed in a solution containing 10 mM H_2O_2 and 3.3 units/mL (0.066 μg of protein/mL) catalase in the presence or absence of PBE. The decomposition of H_2O_2 was followed by the decrease in the absorbance at 240 nm. Catalase activity was expressed as the rate constant of the first-order reaction (*k*) (Aebi, 1984).

Polyacrylamide Gel Electrophoresis (PAGE) and Enzyme Activity Staining. Discontinuous PAGE was carried out under either denaturing or non-denaturing conditions, essentially as described by Laemmli for denaturing condition (Laemmli, 1970). In non-denaturing gel electrophoresis, sodium dodecyl sulfate (SDS), reducing agent (2-mercaptoethanol), and stacking gel were omitted. Gel dimensions and thickness were 14 × 14 and 1.5 mm, respectively. GO (1.3 μg of protein) was subjected to PAGE under either non-denaturing or denaturing conditions on a 10% gel in the presence and absence of PBE. SOD (35 units, 10 μg of protein) and catalase (10 μg of protein) were subjected to PAGE at 4 °C under non-denaturing conditions on a 10% gel and under denaturing conditions on a 14% gel in the presence and absence of PBE. XO (9.25−12.6 μg of protein) was subjected to PAGE under non-denaturing conditions on a 3−8% NuPAGE precast gel and under denaturing conditions on a 10% gel in the presence and absence of test compounds. The mixtures of enzymes and test compounds were prepared in 0.1 M phosphate buffer, pH 7.4, containing 0.1 mM EDTA, and loaded into the wells in a final volume of 20 μL. Following electrophoresis, gels were stained either for activity or protein. Protein staining of the gels was carried out according to the method of Laemmli (1970); however, the acid fixation step was omitted.

XO Activity Staining. Activity staining on polyacrylamide gels was carried out at room temperature. The reaction mixture contained 50 mM Tris-HCl, pH 7.6, 0.5 mM xanthine, and 0.25 mM nitroblue tetrazolium (NBT) (Ozer et al., 1998). Staining of the gels was continued until the activity band(s) appeared on the gels (4−7 min). After the appearance of the bands, gels were washed five to six times with distilled water and scanned.

Superoxide Dismutase (SOD) Activity Staining. SOD activity staining was carried out according to the method of Flohe and Otting (1984) with a minor modification. Immediately after termination of the electrophoresis, gels were soaked in a staining solution containing 0.01% riboflavin, 0.02% EDTA, 0.025% NBT, and 50 mM Tris buffer, pH 7.6, for 10 min. The gels were then exposed to UV light for 15 s. Because the formazan dye is insoluble and remains in the gel matrix, excess reagents were removed by washing the gels with water. The gels were then dried and scanned.

Gel Filtration Chromatography. Gel filtration chromatography of XO and XDH was performed on 2.5 mL bed volume Sepharose 6B columns using 0.1 M phosphate buffer, pH 7.4, containing 0.1 mM EDTA as the equilibration buffer. XO (500 milliunits), with or without 100 μg of PBE, and XDH (62 milliunits), with or without 25 μg of PBE in the equilibration buffer, were applied to the chromatographic column. The enzymes were eluted by passing the same buffer through the column, and 100 μL fractions were collected. XDH and XO activities were measured with 150 μM xanthine by following

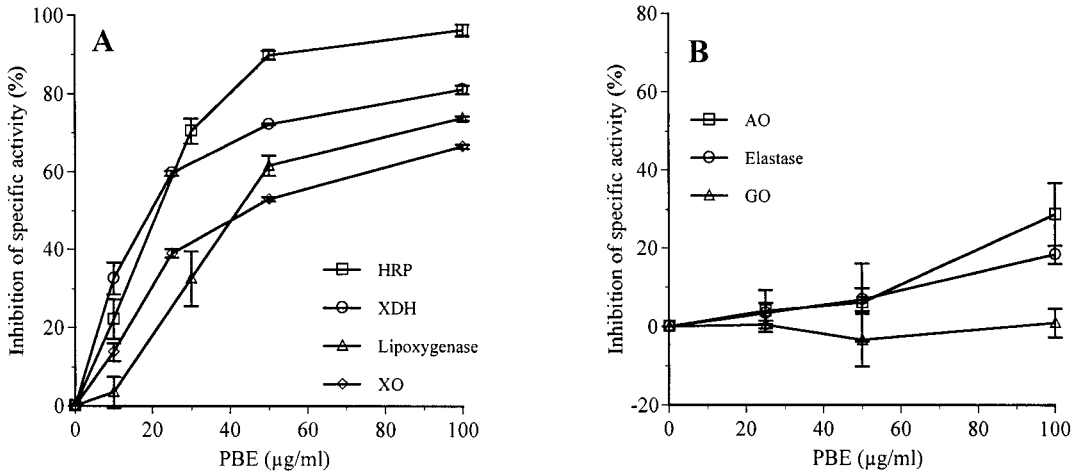

Figure 1. Effect of PBE on enzyme activities. (A) Dose−response curves show the inhibition of lipoxygenase, HRP, XO, and XDH activities as a function of PBE concentration. (B) GO, AO, and elastase activities were not affected by PBE. All enzyme activities were measured as described under Materials and Methods. The enzyme inhibition by PBE was expressed as the percentage of inhibition of specific activity relative to 0% inhibition of the specific activity in the absence of the PBE.

uric acid production at 295 nm in the presence or absence of 500 μM β-NAD$^+$, respectively.

Superoxide Anion Assay. Superoxide anions were generated by UV irradiation of riboflavin/EDTA (Zhao et al., 1989). The reaction mixture containing 0.2 mM riboflavin, 0.4 mM EDTA, and 0.2 M DMPO in the presence and absence of 1 μg/mL of various flavonoids was irradiated with an ultraviolet lamp for 1 min and then transported to a quartz capillary, which was inserted into the cavity of the IBM ER 200D-SRC ESR spectrometer. ESR spectra were recorded at room temperature as follows: central field, 3475 G; modulation frequency, 100 kHz; modulation amplitude, 2 G; microwave power, 10 mW; scan width, 200 G; gain, 6.3×10^5; temperature, 298 K. The scavenging effect of flavonoid on superoxide radical was calculated as $E = [(h_0 - h_x)/h_0] \times 100\%$, where h_x and h_0 were the ESR signal intensities of samples with and without flavonoid, respectively.

Hydroxyl Radical Assay. Hydroxyl radicals were generated by UV photolysis of NP-III and trapped by DMPO (Matsugo et al., 1995). A mixture containing 14 μM NP-III and 0.2 M DMPO, in the presence and absence of 100 μg/mL of various flavonoids, was irradiated by UV lamp for 1 min. The solution was transferred to a capillary tube and then placed in the cavity of the ESR spectrometer. ESR spectra were recorded at 1 min. ESR spectroscopy settings and the calculation of results were the same as in the superoxide anion assay.

Preparation of PBE, EGb 761, and Pure Flavonoids. A stock solution of 10 mg/mL of procyanidins B1, B2, B3, C1, and C2 was prepared in PBS. A stock solution of 100 mg/mL of taxifolin and ECG was prepared in DMSO. A stock solution of 10 mg/mL of PBE, EGb 761, and other pure flavonoids was prepared by dissolving those compounds first in 50 μL of DMSO and then diluting to 1 mL with phosphate buffer, pH 7.4, containing 0.1 mM EDTA.

Protein Determination. Protein was quantitated according to the bicinchoninic acid assay using the Pierce kit (Rockford, IL). Bovine serum albumin was used as standard.

Data Presentation. Data in figures are the mean ± standard deviation (SD) of at least three different experiments performed in triplicate. PAGE was repeated at least three times for each sample with duplicate applications. In some experiments inhibition of the enzyme activities by test compounds was expressed as the percentage of inhibition of specific activity (I) relative to 0% inhibition of specific activity in the absence of the test compound. This was calculated as $I = [(A_c - A_x)/A_c] \times 100$, where A_x and A_c are the specific activities of the enzyme in the presence and absence of test compound, respectively.

RESULTS

The activities of XO, XDH, HRP, and lipoxygenase were measured as 885.4 ± 9.9 nmol of uric acid produced min^{-1} (mg of protein)$^{-1}$, 673.6 ± 5.1 nmol of β-NAD$^+$ reduced min^{-1} (mg of protein)$^{-1}$, 83.4 ± 3.31 μmol of tetraguaiacol produced min^{-1} (mg of protein)$^{-1}$, and 40.9 ± 3.1 μmol of O_2 consumed min^{-1} (mg of protein)$^{-1}$, respectively. PBE at concentrations between 10 and 100 μg/mL inhibited the activity of these enzymes (Figure 1A). GO, AO, and elastase activities were measured as 87.5 ± 1.5 μmol of O_2 consumed min^{-1} (mg of protein)$^{-1}$, 52.3 ± 5.15 μmol of O_2 consumed min^{-1} (mg of protein)$^{-1}$, and 1.9 ± 0.049 μmol of p-nitroaniline produced min^{-1} (mg of protein)$^{-1}$, respectively. However, PBE did not affect the activity of any of these enzymes (Figure 1B).

To characterize the mechanism of PBE action, XO was chosen as an affected enzyme and GO as an unaffected enzyme by PBE. These selections were made on the basis of the high stability of these enzymes and the ease with which their activity can be determined.

To evaluate XO and XDH inhibition by PBE, a steady-state analysis of XO and XDH activities was performed in which the concentrations of xanthine and PBE were varied systematically. It was found that PBE is an uncompetitive inhibitor of both XO and XDH with respect to xanthine as substrate; the lines at different concentrations of PBE in the Lineweaver−Burk plot are parallel (Figure 2). The type of inhibition observed in the Lineweaver−Burk plot was also confirmed by applying Cornish−Bowden and Dixon plots (not shown). The plot of the y-axis of the Lineweaver−Burk intercept versus PBE concentration (Figure 2A,B, insets) defined apparent K_i values of 36.9 and 20.4 μg/mL for XO and XDH, respectively. In the absence of PBE, apparent K_m values of 1.8 and 2.8 μM were observed for XO and XDH, respectively, which approximate the literature K_m value with xanthine as substrate (Greenlee and Handler, 1964; Saito and Nishino, 1989).

To determine whether PBE exclusively interacts with the enzyme−substrate complex as suggested by the results presented above, a second set of steady-state experiments with XDH was performed in which xanthine concentration was held constant and the concentration of β-NAD$^+$ was varied at a series of fixed

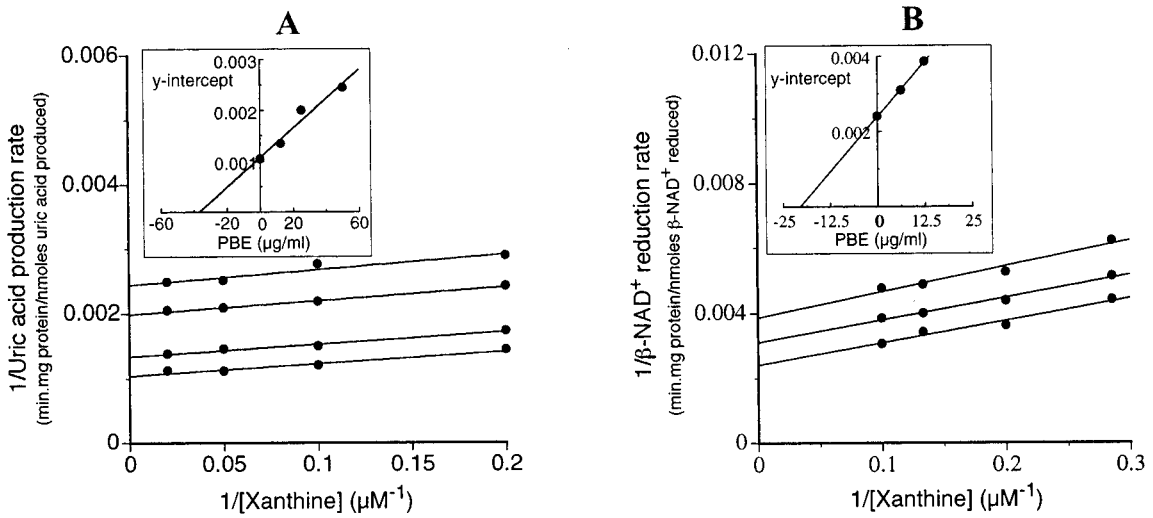

Figure 2. Steady-state analysis of XO and XDH inhibition by PBE. (A) XO activity was measured by following uric acid production at 295 nm. The reaction mixture contained 6 milliunits/mL XO. Xanthine concentration varied at a series of fixed concentrations of PBE; results are presented as a Lineweaver−Burk plot and show an uncompetitive type of inhibition. PBE concentrations used were (from bottom to top) 0, 12.5, 25, and 50 μg/mL. The inset is a secondary plot of *y*-intercept versus PBE concentration. A K_i value of 36.9 μg/mL was estimated for the inhibition of XO by PBE. (B) XDH activity was measured by following the reduction of β-NAD$^+$ at 340 nm. The reaction mixture contained 3 milliunits/mL XDH, 500 μM β-NAD$^+$, and variable concentrations of xanthine and PBE. Results are presented as a Lineweaver−Burk plot and show an uncompetitive type of inhibition. PBE concentrations used were (from bottom to top) 0, 6.25, and 12.5 μg/mL. The inset is a secondary plot of *y*-intercept versus PBE concentration. A K_i value of 20.4 μg/mL was defined for the inhibition of XDH by PBE.

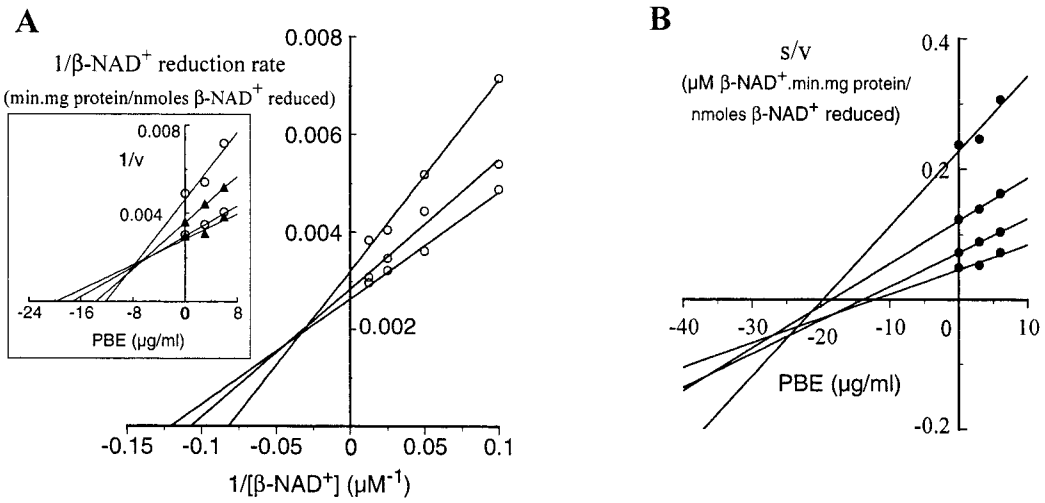

Figure 3. Effect of β-NAD$^+$ concentration on XDH inhibition by PBE. (A) XDH activity was measured by following the reduction of β-NAD$^+$ at 340 nm. The reaction mixture contained 3 milliunits/mL XDH, 150 μM xanthine, and variable concentrations of β-NAD$^+$ and PBE. Results are presented as a Lineweaver−Burk plot. PBE concentrations used were (from bottom to top) 0, 3, and 6 μg/mL. Using a Dixon plot, a K_i value of 6.9 μg/mL was estimated by calculating the median of all intersections (inset). The lines in this plot represent β-NAD$^+$ concentrations of (from bottom to top) 80, 40, 20, and 10 μM. (B) Cornish−Bowden plot of the same experimental data as in (A). The plot shows a mixed type inhibition pattern with respect to β-NAD$^+$. The lines in this plot represent β-NAD$^+$ concentrations of (from bottom to top) 10, 20, 40, and 80 μM.

concentrations of PBE. It was found that the inhibition of XDH by PBE is an intermediate between competitive and noncompetitive, as the intersection of the lines at the different PBE concentrations does not fall on either axis in the Lineweaver−Burk plot (Figure 3A). A K_i value of 6.9 μg/mL was estimated from the Dixon plot (Figure 3A, inset) by calculating the median of all intersections. By applying a Cornish−Bowden plot (Figure 3B), it was confirmed that PBE is a mixed type inhibitor of XDH with respect to β-NAD$^+$ as substrate, because the lines at the different β-NAD$^+$ concentrations intersect under the *y*-axis. It should be pointed out that the K_m value for β-NAD$^+$ of 8.3 μM obtained from the steady-state data at zero PBE concentration is in good agreement with reported values (Saito and Nishino, 1989).

To find out whether PBE directly interacts with the enzyme, XO and GO in the presence and absence of PBE were subjected to PAGE under either non-denaturing or denaturing conditions. Under non-denaturing conditions, the presence of PBE with XO caused the appearance of a band with a slower electrophoretic mobility in the top part of the gel, indicating that PBE changes the electrophoretic mobility of XO (Figure 4A). However, PBE, even in large excess, did not change the electrophoretic mobility of GO (Figure 4B). The presence of SDS (2%) and the reducing agent in the sample buffer did not interfere with the binding of PBE to XO under

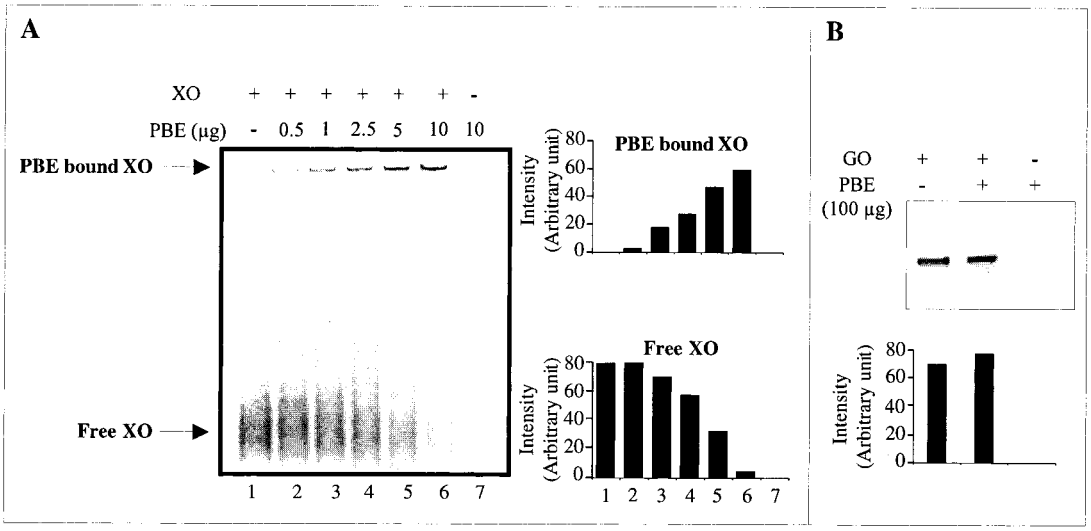

Figure 4. Effect of PBE on the electrophoretic mobility of XO and GO. (A) XO (12.86 μg of protein), PBE, and a mixture of XO and PBE at indicated concentrations were subjected to PAGE under non-denaturing condition on a 3−8% NuPAGE precast gel. Samples were detected by activity staining. The presence of PBE changed the electrophoretic mobility of XO as revealed by a retarded band in the top part of the gel, indicating that PBE binds to XO. The intensity of PBE-bound XO and free XO bands is shown in the right panel. (B) GO (1.3 μg), PBE, and a mixture of GO and PBE were subjected to PAGE under non-denaturing condition on a 10% gel. Samples were detected by protein staining. No GO was detected in the upper part of the gel. A large excess of PBE did not change the electrophoretic mobility of GO. The intensity of the GO band in the presence and absence of PBE is shown at the bottom.

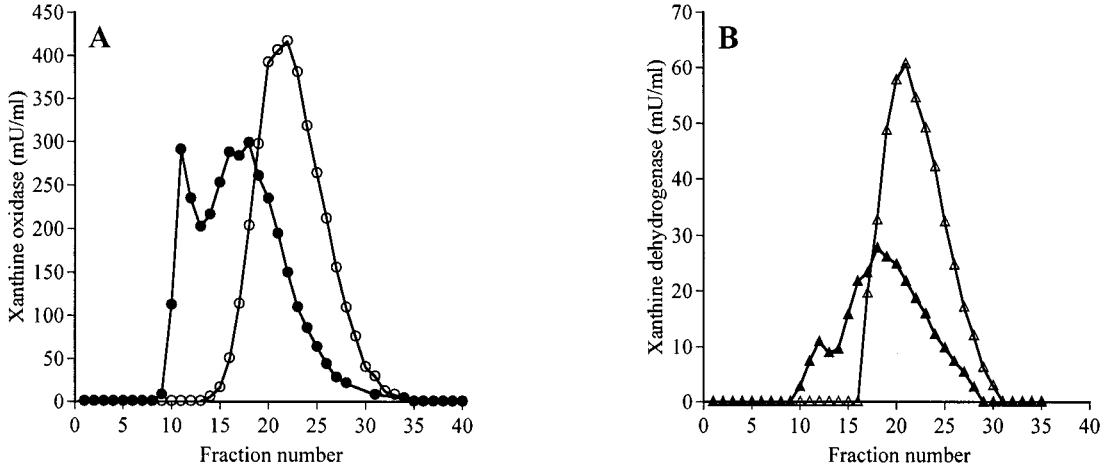

Figure 5. Elution profile of XO and XDH in the presence and absence of PBE. (A) XO (500 milliunits) (○) and a mixture of XO and 100 μg of PBE (●) or (B) XDH (62 milliunits) in the presence (▲) and absence (△) of PBE (25 μg) were applied to gel filtration chromatography. The enzyme activities were measured as described under Materials and Methods and expressed as milliunits per milliliter of eluents. The elution profile of XO and XDH in the presence of PBE shows two peaks representing PBE bound XO and a mixture of free XO and PBE-bound XO.

non-denaturing conditions (data not shown). On the other hand, the electrophoretic mobility of XO and GO was not changed by PBE under denaturing conditions (data not shown).

When applied to gel filtration chromatography, the elution profile of XO in the presence of PBE revealed two peaks (Figure 5A). The first peak represents PBE-bound XO, which corresponds to the retarded band in Figure 4A, whereas the second peak represents a mixture of free XO and PBE-bound XO. Gel filtration of XDH in the presence of PBE exhibited an elution profile similar to that of XO (Figure 5B).

To determine the type of interactions involved in the binding of PBE to XO, XO together with PBE was subjected to PAGE in the presence and absence of agents that compete for ionic and hydrophobic interactions and hydrogen bonding (Figure 6A). When XO and PBE were electrophoresed in the presence of NaCl or urea, the changed electrophoretic mobility of XO could not be restored. However, Triton X-100 dose-dependently restored the changes in electrophoretic mobility of XO as observed by the disappearance of the retarded band. Reversal of the inhibition of XO by PBE was also observed by quantitative measurements of XO activity in the presence of various concentrations of Triton X-100 (Figure 6B). Additionally, polyethylene glycol 400 (PEG 400) has dose-dependently reversed the inhibition of XO by PBE (data not shown).

The antioxidant activities of some purified monomeric flavonoids as well as dimer and trimer procyanidins and their effects on XO activity were compared with PBE. In terms of superoxide and hydroxyl radical scavenging activity, taxifolin was found to be the most potent antioxidant, whereas an extract from *Ginkgo biloba*

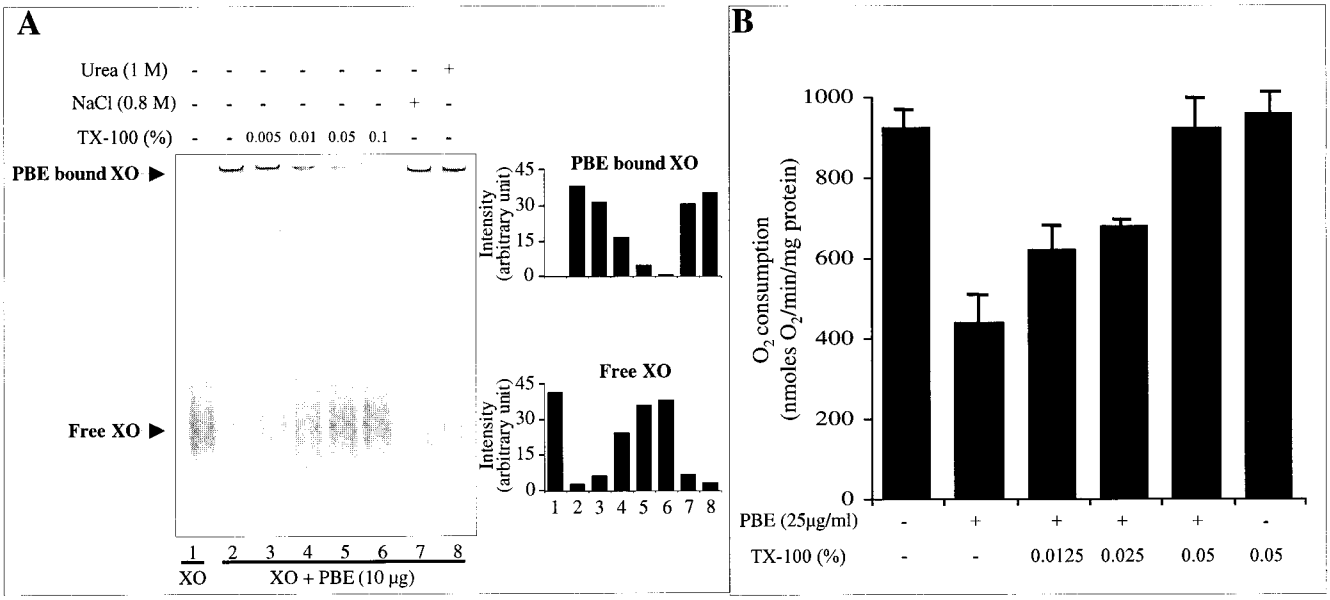

Figure 6. Effect of NaCl, urea, and Triton X-100 on the binding of PBE to XO. (A) XO (12.86 μg of protein) alone or a mixture of XO and PBE was subjected to non-denaturing PAGE in the presence and absence of indicated concentrations of NaCl, urea, or Triton X-100 (TX-100). The concentrations of the agents were calculated according to 20 μL of total loading volume. Samples were detected by activity staining. The retarded band disappears with increasing concentrations of Triton X-100. The intensity of PBE-bound XO and free XO bands is shown in the right panel. (B) Reversal of XO inhibition by Triton X-100. XO activity was measured polarographically in the presence and absence of different concentrations of Triton X-100 at 30 °C as described under Materials and Methods. Urea, NaCl, or TX-100 alone had no effect on either the electrophoretic mobility or the activity of XO.

leaves (EGb 761) showed the lowest superoxide and hydroxyl radical scavenging activities. It is important to note that a minor increase in the production of hydroxyl radical was observed with procyanidins B1, B2, B3, and C1 (Figure 7A).

Epicatechin gallate (ECG) and EGb 761 inhibited XO activity, whereas taxifolin, epicatechin, catechin, and procyanidins C1 and C2 had no effect (Figure 7B). It is worth mentioning that a small increase in the activity of XO was observed in the presence of procyanidins B1, B2, and B3. However, when compared with their antioxidant activity, no correlation was found between the superoxide/hydroxyl radical scavenging activity of the selected flavonoids and their inhibitory effect on XO activity.

Finally, the effect of PBE on the activity and electrophoretic mobility of SOD and catalase was tested. SOD activity was determined in the gel by UV irradiation of riboflavin/EDTA. Electrophoresis was terminated after a predetermined time in which applied PBE in the reference lane had migrated out of the gel. Therefore, no superoxide scavenging activity was observed when PBE alone was applied. Under such conditions, addition of up to 100 μg of PBE did not change either the electrophoretic mobility or the superoxide scavenging activity of SOD. However, PBE dose-dependently inhibited the activity and changed the electrophoretic mobility of catalase under non-denaturing conditions (Figure 8B). When PAGE was performed under denaturing conditions, PBE did not affect the electrophoretic mobility of either SOD or catalase (data not shown).

DISCUSSION

The results of this study suggest that a procyanidin-rich extract binds to the native structure of XO and inhibits its activity and that upon dissociation the enzyme recovers its activity. It appears that binding to the enzyme is the key step in the inhibition of XO activity by PBE.

Effect of PBE on Enzyme Activity. The effect of PBE on enzymes that catalyze the oxidation of their substrate by molecular oxygen and hence generate either intermediate or end product-reactive oxygen species in their enzymatic reaction was investigated. It was found that PBE inhibits XO, HRP, and lipoxygenase activity. GO and AO also catalyze the oxidation of their substrate by oxygen, whereas elastase catalyzes the hydrolysis of peptide bonds at the carboxyl terminal of neutral aliphatic amino acids. However, the activities of these enzymes were not affected by PBE, suggesting that, overall, the effect of PBE on the enzyme activities is selective.

Steady-state analysis of XO and XDH activities demonstrated that PBE is an uncompetitive inhibitor of both XO and XDH with respect to xanthine as substrate, suggesting that PBE can bind only to the enzyme−substrate complex and inhibit the enzyme activity. However, it is known that steady-state kinetics of both XO and XDH reveals a pattern of parallel lines in the Lineweaver−Burk plots, as one substrate concentration is varied at a series of fixed concentrations of the other substrate, indicating a Ping-Pong steady-state mechanism (Olson et al., 1974; Saito and Nishino, 1989). Therefore, the uncompetitive type of inhibition with respect to xanthine could be due to an inhibition in the reduction of the electron acceptors, molecular O_2 and β−NAD^+. Indeed, steady-state experiments with XDH have shown that PBE is a mixed type inhibitor with respect to β-NAD^+ as substrate, indicating that PBE inhibits the reduction of β-NAD^+ to β-NADH that is observed as uncompetitive inhibition with respect to xanthine as substrate. Although due to the lack of necessary devices to change the concentration of molecular O_2 in the reaction mixture, the type of inhibition of XO with respect to O_2 by PBE could not be directly

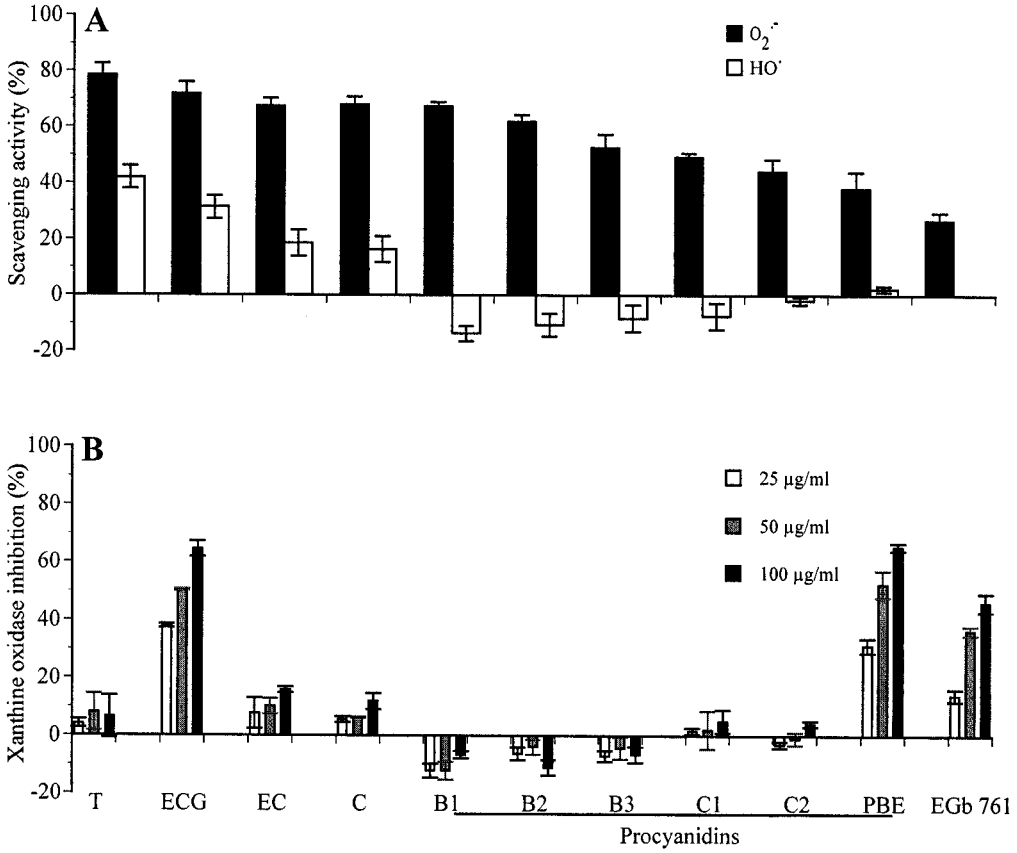

Figure 7. Comparative effect of purified flavonoids and a mixture of flavonoids on XO activity and their antioxidant activity. (A) Superoxide and hydroxyl radical scavenging effect of flavonoids are graphed. Superoxide radicals were generated in the presence and absence of 1 μg/mL of various flavonoids by UV irradiation of riboflavin/EDTA. Hydroxyl radicals were generated in the presence and absence of 100 μg/mL of various flavonoids by UV photolysis of aqueous solution of NP-III. (B) Effect of selected flavonoids on XO activity is graphed. XO activity was measured spectrophotometrically except in the presence of taxifolin, which was measured polarographically as described under Materials and Methods. T, taxifolin; ECG, epicatechin gallate; EC, epicatechin; C, catechin.

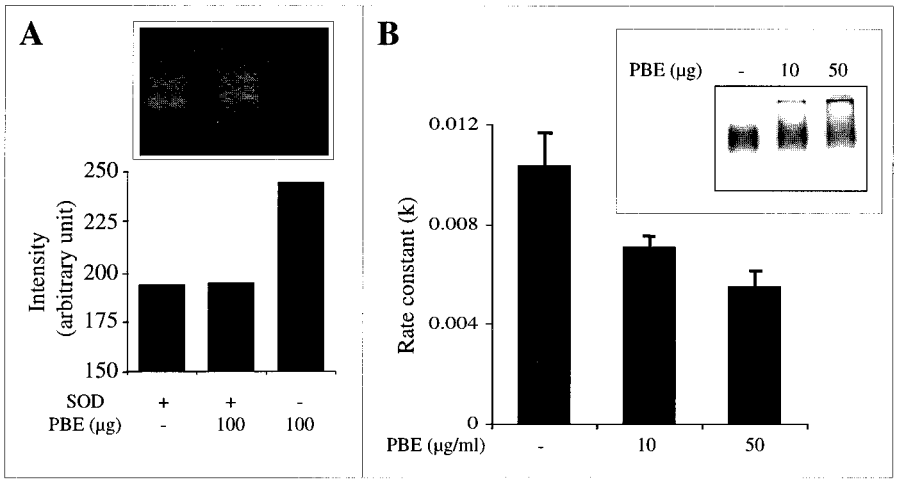

Figure 8. Effect of PBE on the activity and electrophoretic mobility of SOD and catalase. (A) SOD (10 μg of protein) in the presence and absence of PBE was subjected to non-denaturing PAGE at 4 °C on a 10% gel. Samples were detected by activity staining. No superoxide scavenging activity was detected in the upper part of the gel. (B) Catalase activity was measured by following the decomposition of H_2O_2 at 240 nm. (Inset) Catalase (10 μg of protein) in the presence and absence of PBE was subjected to PAGE under non-denaturing conditions on a 10% gel. Samples were detected by protein staining.

assessed; however, it is expected that a mixed type of inhibition of XO occurs with respect to molecular O_2 by PBE.

Binding of Proteins by PBE. The application of PAGE enabled direct determination of the binding of a complex mixture of flavonoids to a particular protein. In the presence of PBE, the electrophoretic mobility of XO under non-denaturing conditions was changed. Under non-denaturing conditions, the movement of proteins through the polyacrylamide gel matrix depends on their size, shape, and charge; thus, a change in the electrophoretic mobility of XO in the presence of PBE could be due to the change in one or more of these parameters. Alternatively, the change in the electro-

phoretic mobility of XO could be due to complexation of XO by PBE. This phenomenon is also consistent with the gel chromatographic results showing higher molecular weight complexes of XO in the presence of PBE. Nevertheless, these results show that PBE binds to XO. It is important to note that because XO was detected by its activity in the gel and in the chromatographic fractions, PBE-bound XO is enzymatically active and, therefore, is not denatured.

The most significant structural difference between XO and XDH is the protein conformation in the 40 kDa domain of the enzyme around FAD, which changes the redox potential of the flavin and the reactivity of FAD with the electron acceptors, NAD and molecular oxygen (Saito and Nishino, 1989). Binding of PBE to XDH suggests that putative binding sites for PBE are not affected by conversion of XO to XDH. Change in the electrophoretic mobility of catalase under non-denaturing conditions suggests that PBE also binds to catalase. However, because PBE did not change the electrophoretic mobility of XO and catalase under denaturing conditions, it appears that binding is specific for the native structure of the enzyme rather than an unfolded protein structure.

The electrophoretic mobility of GO and SOD was not affected by PBE. Although the present paper does not provide any insight into the possible structural differences that cause the binding of PBE to some proteins but not to others, overall, selectivity is observed for the binding of PBE to the enzymes. By using radioligand−receptor binding assays, it has been shown that procyanidin B2 selectively binds to a single receptor, whereas procyanidin B3 binds to 2 receptors of 16 receptors tested. Procyanidin B4 inhibited ligand−receptor binding for 5 of the 16 receptors tested, showing less selectivity when compared to procyanidins B2 and B3 (Zhu et al., 1997). Further investigation is needed to uncover the characteristics of the putative binding site(s) on different protein molecules.

Type of Interaction Mediates the Binding of PBE to XO. Due to the diversity of molecular structures among procyanidins and the variety of functional groups present in proteins, the interaction types such as hydrogen bonding, ionic, and hydrophobic interactions can in principle take place (Loomis, 1974). In a number of studies, Triton X-100, a competitor for hydrophobic interactions, has been used to identify the types of interactions between proteins and cells, phospholipids, and peptides (Klappa et al., 1997; Skogh et al., 1983; Yoshida et al., 1989). Triton X-100, but not urea and NaCl, dose-dependently restored the change in the electrophoretic mobility and the activity of XO, suggesting that hydrophobic bonding might be the dominant mode of interaction between PBE and XO. It has been shown that cytochrome *c* adsorbed on a column of Sepharose containing immobilized polyphenols was effectively eluted by anionic and non-anionic detergents, indicating that hydrophobic bonding may be the major mode of interaction between tannin and proteins (Oh et al., 1980; Spencer et al., 1988). Polyethylene glycols (PEGs) are nondetergent competitors of hydrophobic interactions and are extensively used in hydrophobic interaction chromatography (Lee and Lee, 1987; Shibusawa, 1999). PEG 400 has also reversed the inhibition of XO by PBE, confirming the involvement of hydrophobic interactions in the binding of PBE to XO.

Mechanism of PBE Action on XO. Both PBE and XO are redox active. Upon the binding of xanthine to the molybdopterin-containing domain of XO and reduction of Mo(VI), a rapid transfer of electrons occurs from Mo(IV) to the FAD-containing domain through iron−sulfur centers of the enzyme (Saito and Nishino, 1989). Therefore, PBE could inhibit XO either by interfering with intramolecular electron-transfer processes or by binding to the enzyme and changing its conformation. XO activity may also be affected by a combination of these effects. PAGE experiments have shown that PBE binds to XO and the enzyme inhibition occurs upon binding of PBE to XO and that dissociation of the PBE−XO complex restores enzyme activity. Because no correlation was observed between antioxidant activity and XO inhibition of PBE and selected purified flavonoids, the results point to the importance for inhibition of binding to XO rather than interference with the intramolecular electron-transfer processes. Inhibition of the activity and change in the electrophoretic mobility of catalase by PBE also support the importance of binding to the subsequent inhibition. It is worth mentioning that the present paper does not provide evidence for the mechanism(s) of inhibitory action of PBE on HRP and lipoxygenase. Inhibition of these enzymes by PBE could be mediated by direct protein binding and/or redox based mechanisms.

Antioxidant Activity and Inhibitory Effect of Polyphenols on XO. No correlation was observed between the effect of selected flavonoids on XO activity and their antioxidant activity. Although taxifolin, epicatechin, catechin, and procyanidins B1, B2, B3, C1, and C2 did not affect XO activity, ECG and EGb 761 inhibited the activity of XO. It has been reported that taxifolin, catechin, and epicatechin at concentrations similar to those used in this study do not inhibit XO activity (Change et al., 1993; Cos et al., 1998). The mechanism of inhibition of XO by monomeric flavonoids is not clearly understood. However, it is known that the extent of XO inhibition by structurally related flavonoids is quite different (Cos et al., 1998). Further investigation is needed to clarify whether direct protein binding is involved in the inhibition of XO by these compounds.

PBE contains monomer flavonoids such as catechin, epicatechin, and taxifolin. However, the major constituents of PBE are procyanidins, with a degree of polymerization of up to heptamers, which constitute 75% of its weight. PBE also contains phenolcarbonic acids and their glycosides as minor constituents. Therefore, in view of the fact that selected monomer flavonoids as well as dimer and trimer procyanidins were used at the same concentration as PBE, none of these compounds could be primarily responsible for the effect of PBE on XO activity, suggesting that procyanidins with a degree of polymerization of higher than trimer are possibly responsible for PBE action. It has been reported that PBE inhibits angiotensin-converting enzyme (ACE) in vitro. It has been concluded that the mild hypotensive effect observed in Sprague−Dawley rats after intravenous administration of PBE may be related to this effect (Blaszo et al., 1996). Recently, PBE was reported to stimulate endothelial nitric oxide synthetase (eNOS) activity and increase NO levels in isolated rat aortic ring (Fitzpatrick et al., 1998). The ACE inhibitory and eNOS stimulatory effects of PBE were shown to mostly associate with a fraction of PBE, which contains procyan-

idins with a degree of polymerization of higher than trimer (Blaszo et al., 1996; Fitzpatrick et al., 1998). The active components of PBE, which are responsible for these effects on enzyme activities, remain to be elucidated.

Although the absorption and plasma concentration of monomeric flavonoids have been extensively studied, the metabolic fate of procyanidins is largely unknown (Harmand and Blanquet, 1978; Jimenezramsy et al., 1994; Laparra et al., 1977). Bioavailability of oligomeric procyanidins from PBE has been recently studied in humans (Duweler and Rohdewald, 2000). After oral administration of PBE, two metabolites, δ-(3,4-dihydroxyphenyl)-γ-valerolactone and δ-(3-methoxy-4-hydroxyphenyl)-γ-valerolactone, conjugated with glucoronic acid or sulfate, were identified in the urine, indicating that at least a part of the phenolic components of PBE are bioavailable. Some evidence points to the fact that procyanidins, in vivo, might show some of their characteristics that were observed in vitro. Supplementation of cholesterol-fed rabbits with a procyanidin-rich extract increased the antioxidant capacity of the plasma and strongly inhibited elevated malondialdehyde content of the arterial wall (Yamakoshi et al., 1999). Furthermore, when rats were supplemented with ^{14}C-labeled procyanidins, radioactivity was found several times higher in aorta than in lung and liver, indicating that procyanidins or their metabolites might show a propensity for proline-rich tissues (Harmand and Blanquet, 1978; Laparra et al., 1977). However, further in vivo studies are needed to address the bioavailability of procyanidins as well as to identify the characteristics of their metabolites.

Due to its polyphenolic components, PBE is a scavenger of reactive oxygen species and could act as an antioxidant in vivo. However, the data presented suggest that in addition to the redox-based effects, PBE has direct protein binding properties which could also provide a biochemical basis of its action in biological systems.

ABBREVIATIONS USED

AO, ascorbate oxidase; BSA, bovine serum albumin; DMPO, 5,5-dimethyl-1-pyrroline-*N*-oxide; DMSO, dimethyl sulfoxide; DTT, dithiothreitol; (−)-ECG, (−)-epicatechin gallate; EDTA, ethylenediaminetetraacetic acid; EGb 761, extract of *Ginkgo biloba* leaves; ESR spectroscopy, electron spin resonance spectroscopy; GO, glucose oxidase; HRP, horseradish peroxidase; β-NAD$^+$, β-nicotinamide adenine dinucleotide, oxidized form; NBT, nitroblue tetrazolium; NP-III, *N*,*N*′-bis(2-hydroperoxy-2-methoxyethyl)-1,4,5,8-naphthalenetetracarboxylic diimide; PAGE, polyacrylamide gel electrophoresis; PEG, polyethylene glycol; PBE, pine bark extract (Pycnogenol); PBS, phosphate-buffered saline, pH 7.4; SDS, sodium dodecyl sulfate; SOD, superoxide dismutase; Suc-(Ala)$_3$-*p*-nitroanilide, succinyl-(alanine)$_3$-*p*-nitroanilide; XDH, xanthine dehydrogenase; XO, xanthine oxidase.

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