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Oxidative Degradation of Bisphenol A by Crude Enzyme Prepared from Potato

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When crude enzymes prepared from some vegetables and fruits were incubated with bisphenol A (2,2-bis(4-hydroxyphenyl)propane, BPA) at 37 °C, BPA was oxidized by crude enzymes from potato, eggplant, and lettuce. The crude enzyme prepared from potato (*Solanum tuberosum*) had the strongest oxidative activity for BPA. Its optimal temperature and pH were 40−45 °C and 8.0, respectively. More than 95% of BPA was oxidized after the incubation with potato enzyme for 60 min. BPA gave two oxidation products besides insoluble compounds during the oxidation by potato enzyme. The oxidation products were identified to be 4[1-(4-hydroxyphenyl)-1-methyl-ethyl]-benzene-1,2-diol and 4[1-(4-hydroxyphenyl)-1-methyl-ethyl]-benzene-1,3-diol. Enzymatically oxidized BPA lost the estrogen-like activity to enhance the growth of human breast cancer (MCF-7) cells.

KEYWORDS: Bisphenol A; endocrine disrupting chemicals; oxidation; polyphenol oxidase; potato; *Solanum tuberosum*

INTRODUCTION

Recently, alkylphenols such as *p*-octylphenol (*p*-OP), *p*-nonylphenol (*p*-NP), and bisphenol A (2,2-bis(4-hydroxyphenyl)propane, BPA) have been recognized as endocrine-disrupting chemicals to interfere with hormones of animals as well as phthalic esters and styrene. Among alkylphenols, BPA is widely used as a material for polycarbonate resins and epoxy resins, and it may contaminate food from the inner coating of the can and migrate from polycarbonate tableware and plastic packaging (*1*, *2*). Thus, consumers demand the removal and degradation of contaminated BPA from food and water.

Chemical and biological degradation of BPA have been reported by several researchers. Horikoshi et al. reported that nonylphenol polyethoxylate was photodegraded in the presence of titanium dioxide (*3*). Nonylphenol ethoxylate was oxidized by ozone (*4*, *5*) and hydrogen peroxide (*6*).

On the other hand, there are many reports for biologically degrading and polymerizing alkylphenols such as BPA using microorganisms and enzymes. Ronen and Abeliovich (*7*) observed microbial degradation of BPA and tribromophenol by sediments. Lobos et al. (*8*, *9*) reported that Gram-negative aerobic bacteria could degrade BPA and its analogues. Furusawa et al. (*10*) also found microorganisms which could degrade *p*-NP.

We succeeded in oxidizing *p*-OP but not BPA using tyrosinase from mushrooms (*11*). However, Yoshida et al. (*12*) observed that the tyrosinase could oxidize BPA to quinones. Tanaka et al. (*13*) reported that alkylphenols such as *p*-OP and *p*-NP were degraded by fungal laccase. They also found that basidiomycetes (*Tramates* sp.) could reduce the concentration of BPA, *p*-OP, and ethynylestradiol in a rotating reactor (*14*). Fukuda et al. (*15*) also reported that BPA was degraded by laccase from *Trametes villosa*. Hirano et al. (*16*) found that manganese peroxidase from white-rot basidiomycete (*Pleurotus ostreatus*) could degrade BPA. Sakurai et al. (*17*) polymerized BPA using microbial peroxidase from *Corpinus cinereus*. However, it is difficult to apply a microorganism and its enzyme to foods contaminated with BPA from the viewpoint of food hygiene.

Vegetables and fruits are well-known to have polyphenol oxidases (PPOs, EC 1.10.3.1) such as laccase and tyrosinase. Their PPOs may be able to oxidize BPA, *p*-OP and *p*-NP being polyphenol analogues. In this study, we looked for enzymes to oxidize BPA in vegetables and fruits and found that the crude enzyme extracted from potato could oxidatively degrade BPA and reduce the estrogen-like activity.

MATERIALS AND METHODS

Materials. Potato, eggplant, lettuce, Chinese yam, perilla, okra, burdock, banana, pear, apple, grape, peach, and Japanese plum were purchased from local markets in Sendai, Japan. These vegetables and fruits we chose are known to have a high activity of PPO. BPA was purchased from Shigma-Aldrich Japan K.K.(Tokyo, Japan)

Crude Enzyme. Edible portions of vegetables and fruits were homogenized with 10 volumes of cold acetone (−30 °C) and washed with acetone and water to remove endogenous polyphenols and lipids. After filtration with a Whatman no.2 filter paper, the residue was dried under vacuum overnight. The powder was homogenized with 10 volumes of a mixture of 50 mM citric acid and 100 mM phosphate buffer (pH 7.0) for 10 min at 4 °C and then centrifuged at 10 000*g* for 10 min. The supernatant was used as the crude enzyme.

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Oxidation Test. A typical oxidation assay contained 3 mL of 50 mM citric acid and 100 mM phosphate buffer (pH 8.0), 0.6 mM BPA, and 300 μL of enzyme solution. The mixture was incubated for 5−60 min at 37 °C. The oxidation activity of the enzyme was assayed by monitoring oxygen consumed by BPA using a YSI model 5300 biological oxygen monitor (Yellow Springs Instrument Co. Inc., Yellow Springs, OH).

Temperature and pH were varied from 25 to 70 °C and from 6.0 to 9.0, respectively, to get the optimal temperature and pH of potato enzyme.

High-Performance Liquid Chromatography (HPLC) Analysis. Ethyl acetate (3 mL) was added to the reaction mixture to stop the reaction and to extract BPA and its oxidation products, and the mixture was then centrifuged at 2100*g* for 5 min. The supernatant was used for HPLC analysis. The enzymatic oxidation products of BPA were analyzed by HPLC on a Shodex RS pack DE-413 column (4.6 × 150 mm; Showa Denko Co., Tokyo, Japan). The column was eluted with a mixture of acetonitrile and 0.01 M phosphate buffer (pH 3.7) (30:70, v/v) at a flow rate of 1.2 mL/min. The eluent was monitored with a JASCO MD-1515 multiwavelength detector (Tokyo, Japan) at the range of 200−600 nm. The JASCO−BORWIN software was used to qualify and quantify BPA and its oxidation products.

Instrumental Analyses. Oxidation products of BPA were identified by Fourier transform infrared (FT-IR) spectrum, electron-impact mass spectrum (EI-MS), and ^{1}H NMR after isolation with HPLC. FT-IR was monitored with the Nicolet model 410 Impact FT-IR spectrometer. EI-MS analysis was run on the JEOL Automass mass spectrometer (Tokyo, Japan). The ^{1}H NMR spectrum was recorded on a Varian Unity Inova 600 NMR spectrometer (600 MHz) after dissolving samples in $CDCl_3$.

Estrogen-like Activity Assay. Estrogen-like activity of oxidized and unoxidized BPA was assayed by the method using the estrogen-dependent human breast cancer (MCF-7) cell, because BPA is known to enhance the growth of MCF-7 due to an estrogen-like action (*18*). MCF-7 cells were obtained from the Institute of Development, Aging and Cancer, Tohoku University (Sendai, Japan). BPA was oxidized with potato crude enzyme for 15 and 60 min at 37 °C and pH 8.0. The MCF-7 cancer cells were grown in a Dulbecco's modified Eagle's medium base (D-MEM) containing 5% FBS, 1% penicillin and streptomycin in Falcon T-25 cm^2 flasks and maintained at 37 °C in a humidified atmosphere of 5% CO_2. The pH of the medium was maintained at 7.2−7.4. BPA and oxidized BPA were dissolved in dimethyl sulfoxide (DMSO) and then added to the medium at various concentrations of 125 nM (the final DMSO concentration was 0.1%) after a 24-h incubation. The cells were cultured at 37 °C in 5% CO_2 for 5 days. Viable cells were determined by measuring the absorbance at 570 nm after incubation with 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT) as a color reagent at 37 °C in 5% CO_2 for 3 h.

All tests were performed in 3−6 parallel runs. Data are described as means except for the estrogen-like activity test. Statistical analysis was performed for data obtained in the estrogen-like activity test by analysis of variance (ANOVA) and Fisher's PLSD multiple range test with the use of StatView-J4.11. Differences were considered significant with $P < 0.05$.

RESULTS

Crude enzymes prepared from vegetables and fruits were incubated with BPA for 60 min at 37 °C to estimate their oxidation activity for BPA. Figure 1 shows the consumed oxygen curve of BPA during the enzymatic oxidation. When crude enzymes from potato, eggplant, and lettuce were used, the oxygen consumed by BPA was observed. The crude enzyme prepared from potato showed the strongest oxidative activity for BPA among tested samples. Other vegetables and fruits had no ability to oxidize BPA. Therefore, the crude enzyme prepared from potato (*Solanum tuberosum*) was determined for use as an enzyme for degrading BPA.

The optimal pH was investigated for the potato enzyme by varying pH from 6.0 to 9.0 (Figure 2). BPA was incubated with potato enzyme at different pHs for 5 min at 37 °C. The oxidation rate of BPA was highest at pH 8.0, whereas the potato enzyme did not oxidize BPA at pH 6.0 and pH 9.0.

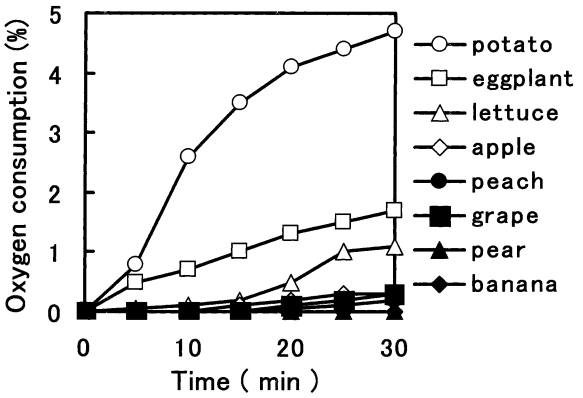

Figure 1. The oxidation of bisphenol A by crude enzymes prepared from vegetables and fruits at 37 °C and pH 8.0.

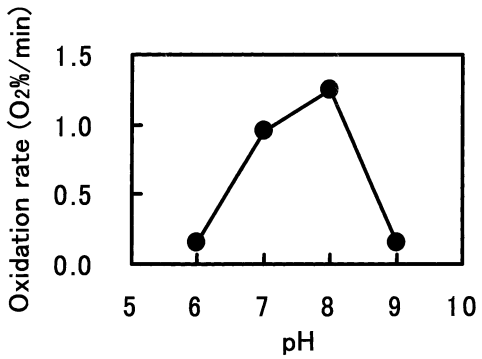

Figure 2. Effect of pH on the oxidation of bisphenol A by the crude enzyme prepared from potato (*S. tuberosum*) for 5 min at 37 °C.

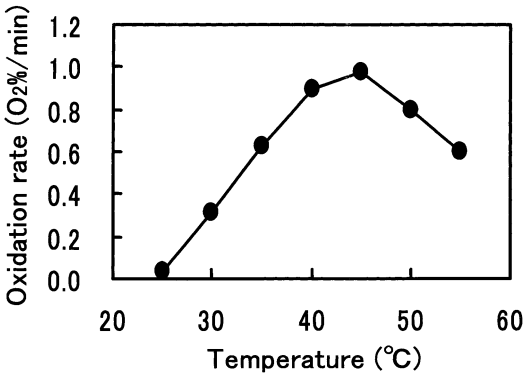

Figure 3. Effect of temperature on the oxidation of bisphenol A by the crude enzyme prepared from potato (*S. tuberosum*) for 5 min at pH 8.0.

The effect of temperature on the oxidation activity of the potato enzyme for BPA was investigated. BPA was hardly oxidized below 25 °C when BPA was incubated with potato enzyme for 5 min at pH 8.0. However, the oxidation rate of BPA increased with the temperature until 45 °C, as shown in Figure 3. The potato enzyme was completely inactivated at 70 °C.

The HPLC chromatogram of BPA after the incubation with potato enzyme for 5 min under optimal conditions is shown in Figure 4. Three peaks were observed at a retention time (Rt) of 27, 31, and 40 min on the HPLC chromatogram. The peak at Rt 40 min was consistent with that of BPA. Thus, the two peaks eluting at Rt 27 and 30 min seemed to be enzymatic oxidation products of BPA. Oxidation products of BPA other than those

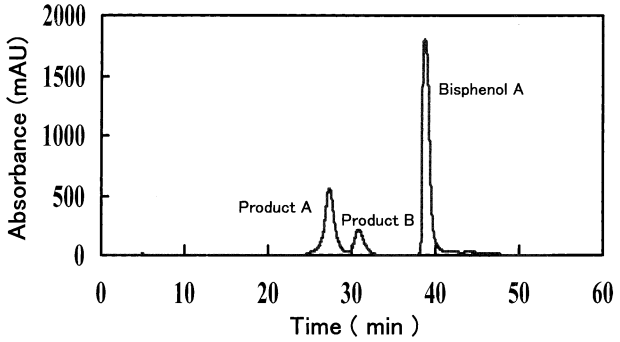

Figure 4. HPLC chromatogram of bisphenol A oxidized by the crude enzyme prepared from potato (*S. tuberosum*) for 5 min at 37 °C and pH 8.0.

two peaks were not detected on the HPLC chromatogram during incubation for 60 min. As for the two peaks, the former (product A) had an absorption maximum at 390 nm, while the absorption maximum of the latter (product B) was 278 nm. When the infrared spectrum was measured for the two oxidation products of BPA, both oxidation products had IR spectra similar to that of BPA. They had an additional OH group (3376 cm^{-1}) as compared to BPA, but they had no oxo group. They also had a benzene nucleus (1610 and 1511 cm^{-1}). When the EI-MS was measured for these oxidation products of BPA, both showed the same spectrum patterns. Products A and B showed a molecular ion peak at *m*/*z* 244 and fragment ion peaks at *m*/*z* 229, 213, 201, 135, 119, 107, 91, 77, 65, and 55. Thus, these oxidation products which had an additional OH group as compared to BPA seemed to be isomers of each other.

The NMR spectrum of product A was slightly different from that of product B. Product A had δ 1.67 (s, 6H, CH_3), 6.71−6.89 (m, 3H), 7.06 (m, 2H), and 7.24 (m, 2H). On the other hand, product B had δ 1.67 (s, 6H, CH_3), 6.53−6.62 (m, 2H), 7.06−7.07 (m, 3H), and 7.24 (m, 2H). From these results, product A was identified as 4[1-(4-hydroxyphenyl)-1-methyl-ethyl]-benzene-1, 2-diol, while product B was 4[1-(4-hydroxy-phenyl)-1-methyl-ethyl]-benzene-1, 3-diol (Figure 5).

Figure 6 shows the changes in the level of BPA and its oxidation products during incubation with the potato enzyme under optimal conditions. BPA decreased with incubation time. More than 95% of BPA was degraded after 60 min. Product A increased until 15 min and then rapidly decreased. On the other hand, product B increased until about 20 min and then gradually decreased. Although the level of product A was higher than that of product B at an initial stage of enzymatic oxidation, the former was less than the latter after 20 min. A considerable amount of product B was residual after 60 min, whereas product A was not detected. These observations suggested that product A being the main enzymatic oxidation product of BPA was unstable and easily changed to product B and polymers (Figure 5).

To investigate the endocrine-disrupting action of oxidized BPA, its estrogen-like activity was measured by estimating the effect on the growth of the estrogen-dependent human breast cancer (MCF-7) cell. When MCF-7 cells were cultured with BPA and oxidized BPA for 5 days, the viable cell numbers were estimated by the MTT method. As shown in Figure 7, BPA enhanced the growth of MCF-7 cells by about 7 times (658%). However, the number of MCF-7 cells incubated with enzymatically oxidized BPA was lower than that with BPA. Especially, the number of MCF-7 cells after incubation with BPA oxidized for 60 min was almost the same (81%) as that of the control cultured without BPA. The level of BPA was reduced to 36

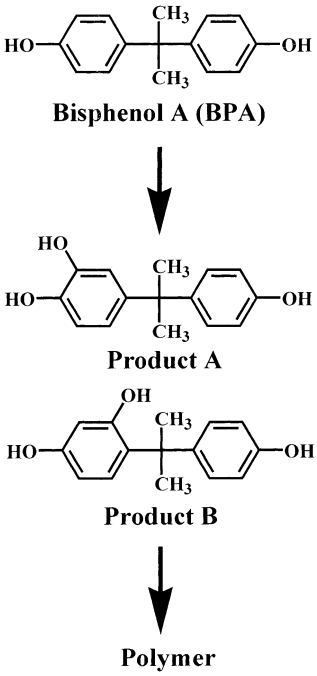

Figure 5. The oxidation of bisphenol A by the crude enzyme prepared from potato (*S. tuberosum*).

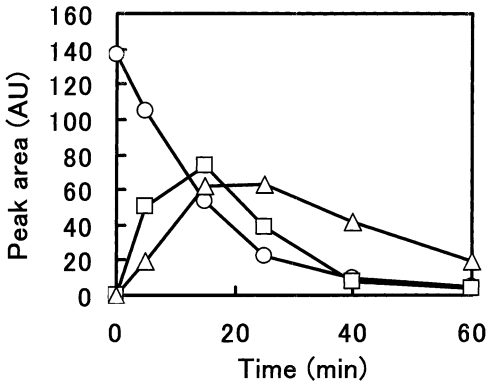

Figure 6. The degradation of bisphenol A during the incubation with potato (*S. tuberosum*) enzyme for 60 min at 37 °C and pH 8.0: ○, BPA; □, product A; △, product B.

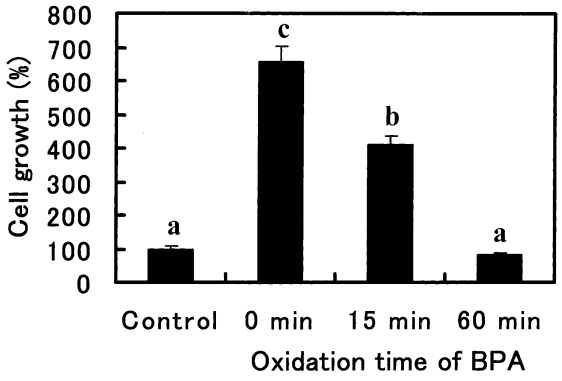

Figure 7. Effect of bisphenol A oxidized with potato (*S. tuberosum*) enzyme on the growth of human breast cancer (MCF-7) cells. Each bar represents the mean ± standard deviation of six samples. Means not followed by a common letter are significantly different ($P < 0.05$).

and 4%, after enzymatic oxidation for 15 and 60 min, respectively. The growth of MCF-7 cells depended on the BPA level. Products A and B might lose the estrogen-like activity because their additional OH group could hinder the combination

with the estrogen receptor on MCF-7 cells. It was suggested that the enzymatically oxidized BPA did not activate the growth of MCF-7 cells because of reduced BPA. From these results, the oxidation with potato enzyme could reduce the estrogen-like activity of BPA.

DISCUSSION

PPOs including monophenol oxidase and diphenol oxidase are enzymes to oxidize mono- and diphenol compounds to quinones, and they are abundant in vegetables and fruits. PPO may oxidize BPA since it is also a polyphenol analogue. Therefore, we investigated the oxidative activity of vegetables and fruits for BPA. As a result, the crude enzyme prepared from potato oxidized BPA. Yoshida et al. (*12*) reported that mushroom tyrosinase could oxidize BPA to the corresponding mono- and bisquinones. In this study, mono- and bisquinones were not detected during oxidation of BPA by potato enzyme, whereas diphenol compounds were produced in addition to polymers. Although the product specificity for BPA of potato enzyme was different from that of mushroom tyrosinase, potato enzyme seemed to be one of the PPOs. Actually, the potato enzyme showed a Michaelis constant (70 μM) similar to that of *p*-cresol (120 μM). Moreover, the potato enzyme may have copper because BPA was hardly oxidized in the presence of L-cysteine and sodium diethyl dithiocarbamate. The enzymatic oxidation of BPA was also inhibited by reductants such as glutathion and ascorbic acid (data not shown).

We also found that BPA oxidized by potato enzyme lost its estrogen-like activity. Probably, the combination ability of BPA with the estrogen receptor on cells might be reduced by enzymatic oxidation. Although many researchers oxidized and degraded BPA using enzymes, their estrogen-like activity has not been measured. In this study, we demonstrated that the enzymatic oxidation of BPA using potato enzyme was effective for reducing the endocrine-disrupting action of BPA.

This potato enzyme is safe in food hygiene because it is edible. Moreover, the oxidative degradation of BPA using the potato enzyme needs no coenzyme. Therefore, the potato enzyme is applicable for degrading BPA in contaminated canned foods. In the next study, we will investigate whether the potato enzyme could effectively oxidize BSA in contaminated foods and drinks.

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