JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Examination of 1,5-Anhydro-D-fructose and the Enolone Ascopyrone P, Metabolites of the Anhydrofructose Pathway of Glycogen and Starch Degradation, for Their Possible Application in Fruits, Vegetables, and Beverages as Antibrowning Agents†

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The anhydrofructose pathway describes the degradation of glycogen and starch to 1,5-anhydro-D-fructose (1,5AnFru) and its further conversion to the enolone ascopyrone P (APP) via the transit intermediate ascopyrone M. The two products, 1,5AnFru and APP, were examined in this study for their effects in controlling the browning of selected fruits, vegetables, and beverages. The results showed that 1,5AnFru had an antibrowning effect in green tea and was able to slow turbidity development in black currant wine. APP proved to be an antibrowning agent comparable to kojic acid. It showed an antibrowning effect in a range of agricultural products, such as various cultivars of apple, pear, potato, lettuce, and varieties of green tea in an efficacy concentration range from 300 to 500 ppm. Mechanism studies indicated that, like kojic acid, APP showed inhibition toward plant polyphenol oxidase and was able to decolor quinones.

KEYWORDS: Anhydrofructose pathway; 1,5-anhydro-D-fructose; antibrowning; ascopyrone P; beverage; fruit juice; green tea; kojic acid; turbidity; vegetable

INTRODUCTION

Miscoloration and decoloration in food products refer to the color changes caused by different processes, such as browning, caramelization, Maillard reaction, degradation of pigments, and oxidation of meat myoglobin. Browning is the miscoloration mainly related to fruits, vegetables, and beverages and is developed in their production processes and storage periods (*1*−*4*). For soft and alcoholic drinks the development of haze and turbidity and the formation of sediments take place usually during storage, processes that may be due to the interactions of oxidized phenols and proteins (*5*). These cause deleterious changes in appearance, other organoleptic properties, and even nutritional value of the finished products, which affect the products' attractiveness and pricing.

The process of browning may involve oxidative enzymes, such as polyphenol oxidase (PPO, EC 1.14.18.1), which occurs in fruits and vegetables (*6*), and other chemical reactions such as the nonenzymatic oxidation and polymerization of plant phenols and quinones. PPOs of plant origin are copper metalloproteins that catalyze the oxidation of mono- and *o*-diphenols to *o*-diquinones. For food producers in this area color stabilization and inhibition of browning are major goals (*7*). Among the fresh-keeping agents, sulfite has long been used in many of these products because of its efficacy as an antibrowning agent, its antimicrobial effect, and its low price (*7*, *8*). It is, however, well-known that sulfite causes public concerns due to its potential health risks, in particular for asthmatics (*1*). This is why in some countries its uses in certain application areas, such as fresh salads, have been banned. A total ban of sulfiting agents has not yet been possible simply because the industries today find no suitable substitute for it. Ascorbic acid and kojic acid show antibrowning properties. For ascorbic acid it is also known that it may promote browning in certain products (*9*). Kojic acid is produced by the fungal species of *Aspergillus* and *Penicillium* (*10*, *11*). Its high price has basically excluded its uses in food areas. To search for new antibrowning agents, we have been screening fungal and algal natural metabolites. Among the promising metabolites we have found are 1,5-

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† This paper is dedicated to Prof. Marianne Pedesén from Stockholm University, Carl von Linné gold medal receiver and ex-superviser to S. Yu, on the occasion of her 70th birthday.

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10.1021/jf0515274 CCC: $30.25
Published on Web 11/08/2005

anhydro-D-fructose (1,5AnFru), a metabolite from both fungi (*12*, *13*) and red algae (*14*, *15*), and ascopyrone P (APP), a metabolite from fungi (*16*). We further succeeded in elucidating the biosynthetic pathway of the two metabolites and in purifying the related enzymes from these organisms (*13*, *14*, *17*, *18*). As a result of this, 1,5AnFru and APP are available in large amounts by biocatalysis using enzyme reactors followed by downstream separations (*18*−*20*).

In the current study, 1,5AnFru and APP were examined for their effects in plant and beverage products with respect to browning control and prevention of turbidity development. In certain examples they were compared with ascorbic acid and kojic acid. It was found that 1,5AnFru had a certain effect in browning control in green tea and control of turbidity development in black currant wine. APP exhibited an antibrowning effect comparable to that of kojic acid. The mechanism of APP in browning control is due to its inhibition toward PPO and the reduction of quinones, the same as kojic acid (*21*, *22*).

MATERIALS AND METHODS

Chemicals. 1,5AnFru and APP were prepared from starch by using the enzymes of α-1,4-glucan lyase (EC 4.2.2.13), 1,5AnFru dehydratase, and ascopyrone tautomerase in our Copenhagen laboratory as described before (*17*, *20*, *23*). Mushroom polyphenol oxidase (EC 1.14.18.1) from *Agaricus bisporus*, DL-dopa (3,4-dihydroxy-DL-phenylalanine), kojic acid, and ascorbic acid were all obtained from Sigma (St. Louis, MO). Other reagents were obtained locally in Denmark and were of analytical grade.

Biological Material. Apple (*Malus domestica*) and pear (*Pyrus communis*, *Pyrus bretschneideri*) were obtained from the local markets in Copenhagen (Denmark) and Laiyang (China) in their harvest seasons. The apple cultivars (cv.) used were Golden Delicious, Fuji Red, Granny Smith, Gloster, Jona Gold, Elstar, Red Gravenstein, and Royal Gala. The pears used were *P. communis* cv. Beurre d'Anjou, Packham's Triumph, and Alexander Lucas and *P. bretschneideri* cv. Laiyangli. Additional samples of apple *Malus baccata* and *Malus orientalis* and pear *Pyrus ussuriensis* var. *hondoensis* were obtained from the botanical garden of the Swedish University of Agricultural Sciences at Alnarp campus (Alnarp, Sweden). The vegetables used were lettuce (*Lactuca sativa*) and potato (*Solanum tuberosum* cv. Bentje). The Sencha, Longjing, and Java green teas (*Camellia sinensis*) and the Darjeeling green tea (*Camellia sinensis* var. *assamica*) were obtained from R. Twining & Co. Ltd. (London, U.K.) and local supermarkets. Black currant wine was provided by Danish Distillers A/S (Copenhagen, Denmark).

Antibrowning Effects of 1,5AnFru and APP in Fruit Products and Green Tea. The fruit and vegetable products were cleaned with water, cut in cubes or pieces, and homogenized in a tissue homogenizer for 2−5 min. The juices obtained were directly mixed with the compounds to be tested, that is, 1,5AnFru, APP, ascorbic acid, and kojic acid. The juices of these compounds were stored at 5 °C from 1 week to up to 4 months and examined periodically. The fruits were sliced and sprayed with a water solution of 1,5AnFru or APP. They were kept at room temperature (22 °C) and were observed visually and recorded photographically. For spectrophotometric measurements the juice was first clarified by centrifugation at 3000*g* for 5 min at 5 °C to precipitate insoluble material.

Green tea was made by soaking the tea leaves in hot water (95−100 °C) for 2−3 min. The hot tea was then added to containers with and without the addition of different amounts of 1,5AnFru (0−1.0%) and APP (0−500 ppm). The containers had headspaces of 30−50% of their volume. Alternatively, the hot tea was first cooled to 22 °C using an ice bath before mixing with 1,5AnFru and APP. The treated tea samples were stored for 1 year at 22 °C in the dark and under fluorescence light with a photon irradiance of 60 μmol m^{-2} s^{-1} and a light/dark regimen of 8/16 h and examined periodically. Color development of the products was recorded by photographing with a digital camera and by measuring chroma difference (ΔE) using a tristimulus colorimeter. The chroma difference (ΔE) was defined as $\Delta E = [(\Delta L)^2 + (\Delta a)^2 + (\Delta b)^2]^{1/2}$.

1,5AnFru in Black Currant Wine Applications. The concentrations tested were 0 (control), 500, and 1000 ppm. The wine samples were stored under the following conditions: in the dark at 4, 22, and 34 °C and under fluorescence light with a photon irradiance of 110 μmol m^{-2} s^{-1} at 22 °C. Sample turbidity was measured as FNU by nephelometry using a NEPHLA turbidimeter. Samples were taken for turbidity and browning analysis at 0, 0.5, 1, 2, 3, 4, 6, 9, and 12 months.

Extraction and Assay of PPO. Apple peel from Fuji Red (0.5 g) was mashed at 5 °C using a mortar and pestle in 5 mL of sodium phoshate buffer (50 mM, pH 6.8). The preparation was left at 5 °C for 1 h, filtered, and centrifuged at 10000 rpm. The supernatant was used as cell-free PPO extract. The PPO assay mixture (3.0 mL) consisted of 0.5 mL of 20 mM catechol (1,2-benzenediol), 0.1 mL of PPO extract or mushrom PPO from Sigma suspended in water, and phosphate buffer in a final concentration of 20 mM (pH 6.8). The reaction was performed at 32 °C and followed spectrophotometrically at 420 nm after a preincubation of the mixture minus PPO at 32 °C for 5 min. One PPO unit was defined as the amount of enzyme that caused an increase of 0.001 absorbance unit at 420 nm per minute. The specific activity was defined as PPO units per gram of fresh weight tissue (units·g^{-1} of FW). In the control, heat-inactivated PPO (100 °C, 5 min) was used.

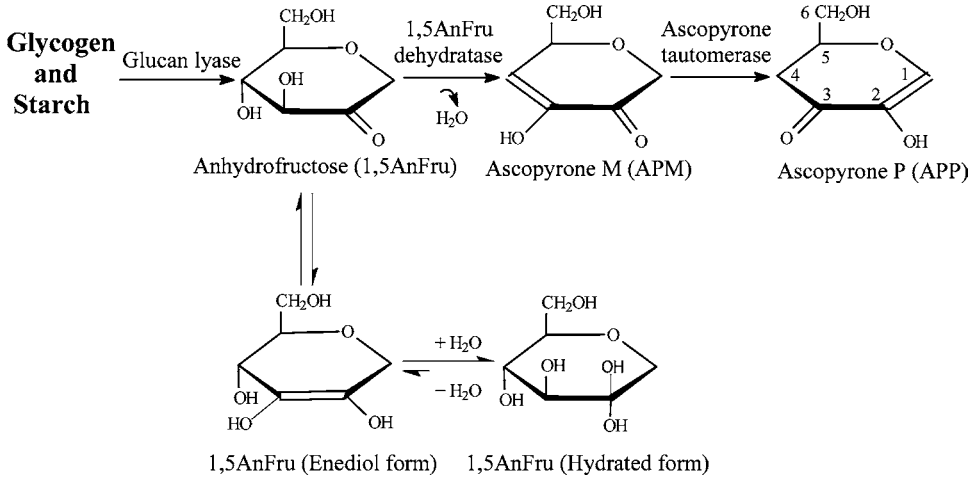
Reduction of Quinone. DL-Dopaquinone was generated from DL-dopa using PPO as the biocatalyst as described before (*22*) with some minor modifications. The PPO-catalyzed reaction was aerated to speed the formation of DL-dopaquinone; the generated reddish dopaquinone was separated from the PPO at the end of the reaction by passing it through a membrane with a 10 kDa cutoff. APP and kojic acid were tested at a final concentration of 4 mM, whereas 1,5AnFru was tested at 20 mM. The reduction of the dopaquinone was followed by scanning the DL-dopaquinone reaction mixture containing APP, kojic acid, or 1,5AnFru between 250 and 700 nm at 1 nm intervals at 22 °C and by monitoring at 480 nm at 5 min intervals over a period of 14 h using a microplate scanning spectrophotometer (model PowerWave$_x$, Bio-TEK Instruments, Inc., Winooski, VT).

RESULTS AND DISCUSSION

Biosynthesis of 1,5AnFru and APP and Structural Comparisons with Ascorbic Acid and Kojic Acid. As shown in **Scheme 1**, 1,5AnFru and APP can be formed from starch and glycogen enzymatically (*17*, *18*). The fungal occurrence of 1,5AnFru and APP was reported more than a decade ago by Baute et al. (*16*). No hypothesis has been proposed for their possible antibrowning effects, and no functional examinations were made on these metabolites. In our Copenhagen laboratory the biosynthetic pathway of APP has recently been elucidated (*17*, *18*), and we have been able to produce 1,5AnFru and APP by enzyme reactor technology (*17*, *20*, 23). A search of chemical databases revealed that the enediol tautomer of 1,5AnFru resembles L-ascorbic acid and D-isoascorbic acid (erythorbic acid) in their common enediol functional group (**Scheme 1**). APP is structurally similar to kojic acid as both metabolites possess a keto−enol conjugate system as their functional group (*10*, *11*) (**Scheme 1**). Because of the structural similarities, 1,5AnFru and APP were here examined for their antibrowning effects and compared with L-ascorbic acid and kojic acid.

Antibrowning Effect of 1,5AnFru in Fruit Products and Green Tea. It was found that at a concentration of 1.0% (w/v), 1,5AnFru had a limited antibrowning effect on apple slices and juices of the cultivars of Golden Delicious, Summer Red, Granny Smith, Elstar, Jona Gold, Gloster, Red Gravenstein, and Royal Gala. At a concentration of 0.3% or lower, 1,5AnFru showed no appreciable effect in controlling the browning of the fruit slices of apple and pear as examined. 1,5AnFru at 1.0% exhibited no substantial browning inhibition effect on slices of *M. baccata* and *M. orientalis* as these slices turned brown in a

Scheme 1. Anhydrofructose Pathway Leading to the Formation of the Antibrowning Agent Ascopyrone P (APP) via 1,5-Anhydro-D-fructose (1,5AnFru) and Ascopyrone M (APM) and the Tautomerization of 1,5AnFru



couple of minutes after cutting. The fast browning of these apple slices was in contrast to the browning of domestic cultivars, which took usually 5−30 min before noticeable browning could be observed.

1,5AnFru was unable to exert any appreciable inhibition toward mushroom PPO from *A. bisporus* at 2 mM (344 ppm) and 20 mM (3440 ppm), whereas under the same conditions L-ascorbic acid gave a complete inhibition at both concentrations as expected (figure not shown).

An effect of 1,5AnFru in browning control was observed from 0.5 to 1.0% (w/v) for all of the green tea varieties tested, that is, Sencha, Longjing, Java, and Darjeeling. Comparing with the control, the addition of 0.1% of 1,5AnFru produced also a clear antibrowning effect (**Figure 1**). The antibrowning effect of 1,5AnFru at 1.0% lasted for at least 1 year when the Darjeeling green tea was kept under light at 22 °C in transparent plastic containers with 40% headspace. Besides the concentrations of 1,5AnFru, the browning of green tea was also related to the headspace volume and storage time, whereas storage in the dark or under light (60 μmol m^{-2} s^{-1}) gave no appreciable differences in browning. In the case of Sencha green tea, the antibrowning effect of 1,5AnFru was examined by mixing it with hot tea and with tea that had been cooled to 22 °C. The results obtained were the same. The relatively high concentrations of 1,5AnFru needed for controlling the browning of green tea may be due to the very low concentrations of the enediol tautomer present in aqueous solution (*15*, *19*) (**Scheme 1**).

Antibrowning and Stabilizing Effect of 1,5AnFru in Black Currant Wine. Black currant wine is known for its browning and development of turbidity during storage. The mechanism for turbidity development is not fully understood. It is assumed to be a result of the interaction among quinones, proteins, and pectin material (*5*). Upon the addition of 1,5AnFru at 500 and 1000 ppm, no noticeable inhibitory effect in browning development could be seen in an observation period of 12 months when the wine was stored in the dark at 22 and 34 °C or under constant light at 22 °C. A brown color was clearly seen after 4 months of storage at 22 and 34 °C. In contrast, refrigerated wine samples at 4 °C showed no color changes in the absence or presence of 1,5AnFru. For the turbidity measurements, it was observed that 1,5AnFru at 500 and 1000 ppm had an enhancing effect in reducing the turbidity development in all three treatments (**Figure 2**). A turbidity reduction by a factor of 3 was seen at 1000 ppm of 1,5AnFru for samples kept under constant light at 22 °C (**Figure 2C**). Storage at 22 °C in the dark (**Figure 2A**) and light (**Figure C**) gave higher turbidity development than that at 34 °C in the dark (**Figure 2B**). 1,5AnFru displayed no effect for the wine samples kept in the dark at 4 °C since the turbidity development under such condition was limited (figure not shown). A periodic organoleptic examination indicated that 1,5AnFru at 500 and 1000 ppm did not affect the odor and taste negatively. These results indicate that 1,5AnFru may be used at 1000 ppm to slow turbidity development in black currant wine, especially if the wine is exposed to light for a longer period.

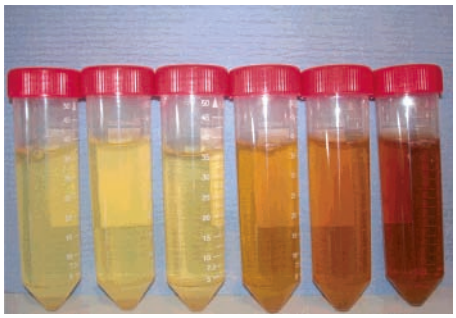


Figure 1. Inhibition of 1,5AnFru on the browning of Darjeeling green tea. To freshly made green tea was added 1,5AnFru to final concentrations (%, w/v) of 1.0, 0.7, 0.5, 0.3, 0.1, and 0 (from left to right) in 50 mL falcon tubes. Photographs was taken after the teas had stood at 22 °C for 2 weeks.

APP as an Antibrowning Agent for Fruit and Vegetable Products. Figure 3 shows the antibrowning effect of APP on Gloster apple juice presented by absorbance spectra. The absorbance values between 350 and 550 nm were higher in the control, due to the brown color formation, than in those samples with the addition of 550 and 2750 ppm of APP. The respective areas (arbitrary units) under the curves were 81.4 for the control and 49.8 and 29.9 for APP at 550 and 2750 ppm, respectively. It was found that APP at 500 ppm was needed to keep the Gloster apple juice from browning when concentrations of 0, 100, 200, 300, and 500 ppm were examined. For Golden Delicious apple juice a concentration of 300 ppm was adequate in controlling the browning (**Figure 4**), and the antibrowning effect was still observable after a storage of 4 months at 5 °C.

For apple slices made from all of the cultivars of *M. domestica*, that is, Golden Delicious, Summer Red, Granny Smith, Elstar, Jona Gold, Gloster, Red Gravenstein, and Royal Gala, APP gave an antibrowning effect in a concentration range

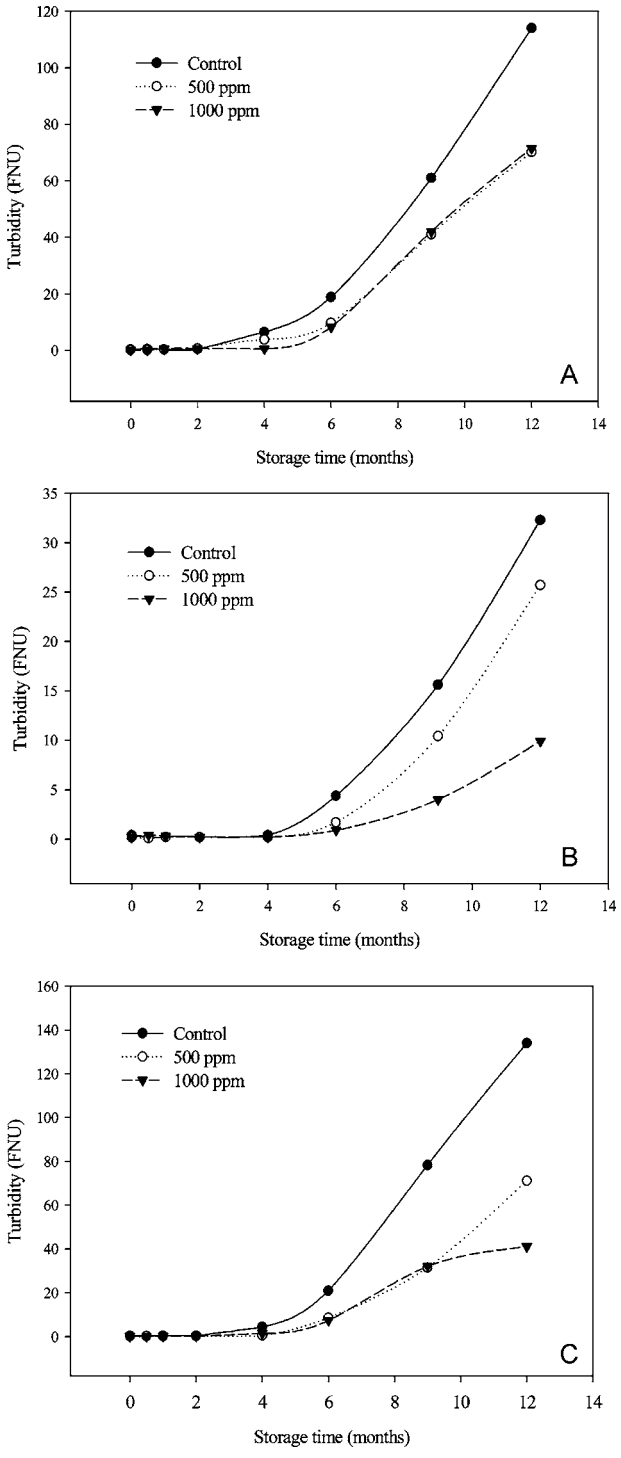


Figure 2. Effect of 1,5AnFru on the turbidity development in black currant wine. 1,5AnFru was added to the wine at final concentrations of 0 (control), 500, and 1000 ppm and mixed, and the wines were stored for 12 months at 22 °C in the dark (**A**), at 34 °C in the dark (**B**), and at 22 °C under constant fluorescence light (**C**). Turbidity development (in FNU) was measured periodically.

from 300 to 500 ppm. For the slices made from *M. baccata* and *M. orientalis*, 1000 ppm was needed for browning control.

For the pear juice made from *P. communis* cv. Beurre d'Anjou, it was found that 200 ppm of APP was needed to keep it from browning when tested at concentrations of 0, 10, 30, 50, 100, 200, 300, and 500 ppm. A similar effect was seen for the pear cultivars Packham's Triumph, Alexander Lucas, and *P. bretschneideri* cv. Laiyangli. APP was also efficient in

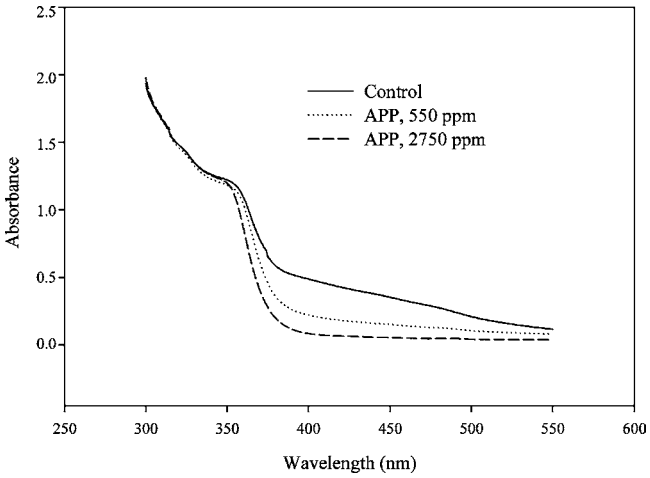


Figure 3. Absorbance spectra of Gloster apple juice in the absence and presence of APP. Freshly made juice with (550 and 2750 ppm) and without the addition of APP was scanned between 190 and 650 nm, and the spectrum window from 300 to 550 nm is shown.

Figure 4. Antibrowning effect of 300 ppm of APP on Golden Delicious apple juice. The photograph was taken right after the juice was made.

controlling the browning of salad lettuce and potato slices with an efficacy concentration range of 300−500 ppm (figure not shown).

APP was also capable of controlling the browning of green tea just like 1,5AnFru, but it was 20−30 times more efficient than 1,5AnFru. APP at 200 ppm was able to keep the original yellowish color of Darjeeling green tea for at least 1 week at 22 °C in the light (60 μmol m^2 s^{-1}) when tested at 0, 30, 50, 100, 200, 300, and 500 ppm. Similar results were seen for Sencha, Longjing, and Java green teas. The effect of APP on the color change of Longjing green tea was further recorded by using a tristimulus colorimeter and compared with kojic acid. **Figure 5** shows that there were no significant chroma differences (ΔE) in newly made green tea irrespective of the presence of various concentrations of APP and kojic acid. With the increase of storage time at 22 °C, the ΔE values for the groups with the addition of APP and kojic acid were significantly lower than those of the control (**Figure 5**). APP gave an effect similar to that of kojic acid at 100 and 200 ppm in controlling the browning of green tea for at least 4 days.

Inhibition of APP on PPO from Apple and Mushroom. On the basis of structural similarity (**Scheme 1**), the browning control mechanism of APP might be similar to that of kojic acid by the inhibition of PPO activity and by reducing quinones to phenols. To prove this hypothesis, APP was examined for its possible inhibition toward PPO from Fuji Red apple and *A. bisporus*. It can be seen from **Figure 6** that APP and kojic acid showed an inhibition toward PPO from the apple peel (**Figure 6A**) and mushroom (**Figure 6B**). The apple peel PPO was inhibited by 500 ppm of APP at an extent comparable to that of kojic acid. APP lower than 500 ppm was much less efficient

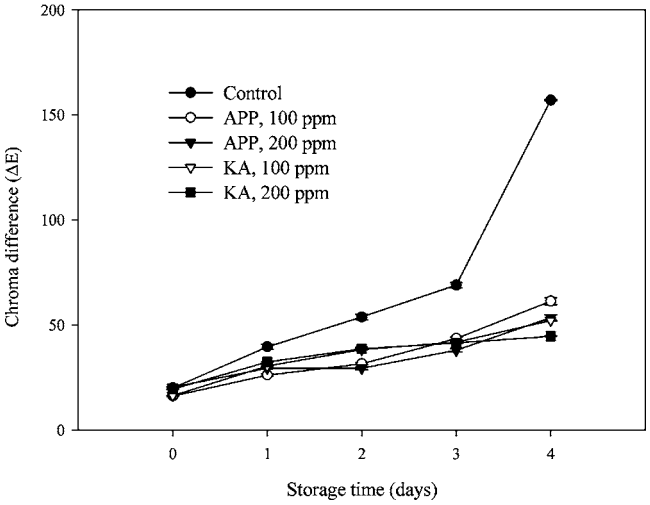


Figure 5. Antibrowning effect of APP and kojic acid (KA) on Longjing green tea. APP and KA were added to the freshly made green teas at 0, 100, and 200 ppm. Chroma difference (ΔE) was measured every day over a period of 4 days using a chroma difference colorimeter.

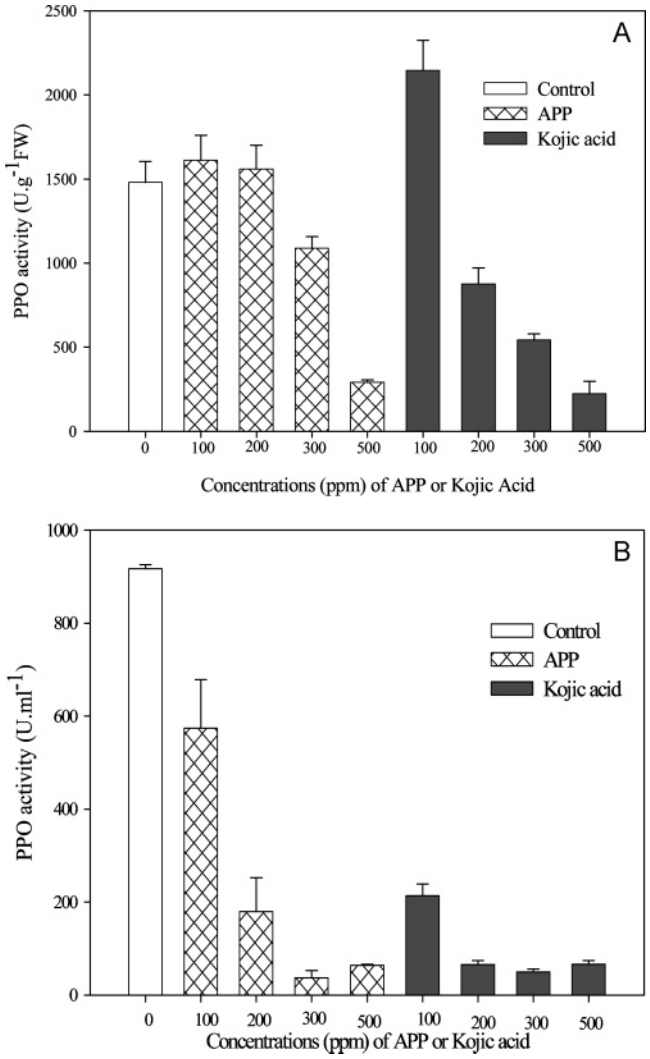


Figure 6. Inhibition of APP on PPO from Fuji Red apple peel (**A**) and mushroom (**B**). APP concentrations tested were 0, 100, 200, 300, and 500 ppm.

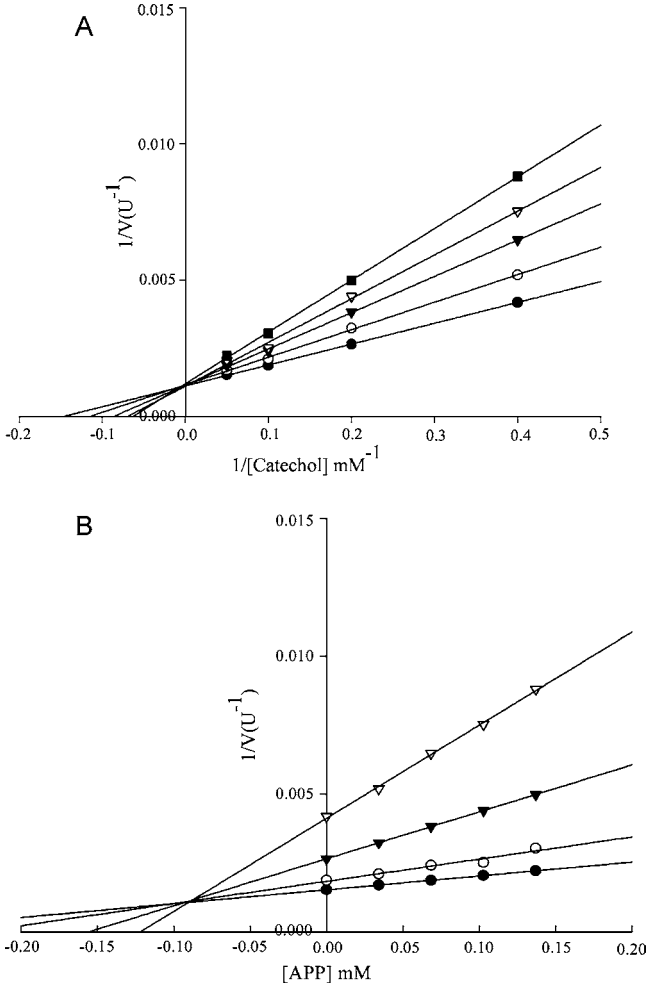


Figure 7. Kinetic studies of APP on mushroom PPO: (**A**) Lineweaver–Burk plot of APP inhibition toward PPO in the presence of APP at 0 (●), 5 (○), 10 (▼), 15 (▽) and 20 (■) ppm; (**B**) Dixon plot of APP inhibition toward PPO at catechol concentrations of 2. 5 (▽), 5 (▼), 10 (○), and 20 (●) mM.

than the corresponding concentrations of kojic acid, except 100 ppm of kojic acid, which showed high values than controls (**Figure 6A**). For the mushroom PPO, APP at 300 and 500 ppm was as efficient as kojic acid (**Figure 6B**). For further mechanism studies the purified mushroom PPO was used. The inhibition type was found to be competitive (**Figure 7A**) as the increase in K_m values was proportional to the increase in APP concentrations. Using catechol as substrate, the K_m determined was 6.8 mM; the inhibition constant (K_i) of APP was estimated to be 93 μM by Dixon plot (**Figure 7B**).

Reduction of Dopaquinone. Figure 8A shows the reduction of DL-dopaquinone by APP as indicated by spectrum scanning. After a longer reaction time the DL-dopaquinone was completely decolored. **Figure 8B** shows the comparisons of APP and 1,5AnFru with kojic acid in their reaction with the quinone. It can be seen that kojic acid at 4 mM was more efficient in decoloring the quinone than 4 mM APP and 20 mM 1,5AnFru as indicated in the fast initial decrease in absorbance at 480 nm. The complete decoloration by APP and 1,5AnFru was reached at ~7 h; thereafter, the absorbance of the reaction mixture remained unchanged, whereas for kojic acid, the quinone solution, which first became completely decolored, turned light green after 2 h of reaction as indicated by the absorbance increase at 480 nm. This is possibly due to secondary reactions. It is known that antibrowning agents may sometimes promote miscoloration and that antioxidants may become prooxidative (*9*). The reducing capability of APP was also seen in

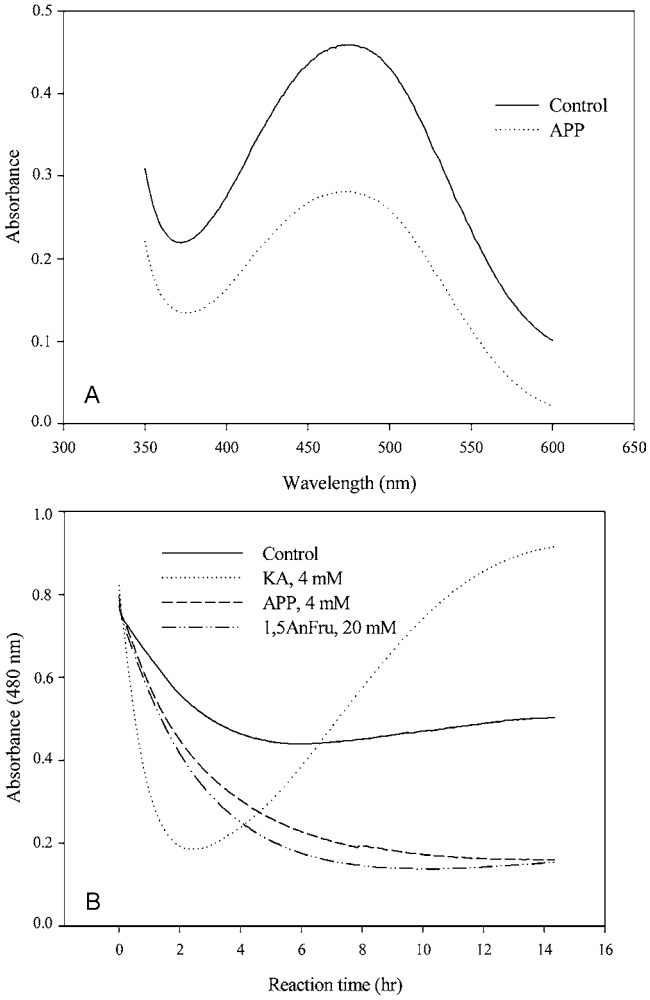


Figure 8. Reaction of DL-dopaquinone with APP, 1,5AnFru, and kojic acid (KA). (**A**) Changes of absorbance spectra of DL-dopaquinone upon its reaction with APP. The absorbance spectra of the DL-dopaquinone solution were recorded in the absence (upper curve) and presence (lower curve) of 4 mM APP, after having been incubated at 22 °C for 40 min. The spectrum from 250 to 700 nm was recorded, and only the spectrum of 350–600 nm is shown. (**B**) Absorbance changes at 480 nm of DL-dopaquinone in the presence of APP, 1,5AnFru, and KA at 30 °C over a period of 14 h.

its reduction (decoloration) of 2,6-dichlorophenolindolphenol (DCPIC) and methylene blue (data not shown).

In summary, 1,5AnFru showed a clear antibrowning effect in green tea at relatively high concentrations of 0.3−1.0% (w/v). The inefficient antibrowning effect of 1,5AnFru may be due to its inability to inhibit PPO and the low concentration of the 2,3-enediol tautomer (*15*). 1,5AnFru was able to slow turbidity development in black currant wine. Our toxicology studies showed that 1,5AnFru was safe with respect to genotoxicity for prokaryotes and eukaryotes (*24*) and safe for rats in a 90-day feeding study at 1 g kg^{-1} of body weight (*25*). APP showed a respectable antibrowning effect in various cultivars of apple, pear, and varieties of green tea. We propose that the mechanism of APP in controlling browning reactions is its inhibition of PPO and the reduction of quinones, the same as kojic acid. Thus, APP is a promising bifunctional agent in the control of browning and bacterial growth (*23*, *26*). The application of 1,5AnFru and APP in green tea, fruit, and vegetable products for human consumption awaits further safety studies, perhaps with clinical trials.

ABBREVIATIONS USED

1,5AnFru, 1,5-anhydro-D-fructose (1,5-anhydro-D-*arabino*-hex-2-ulose); APM, ascopyrone M (1,5-anhydro-D-*glycero*-hex-3-en-2-ulose); APP, ascopyrone P (1,5-anhydro-4-deoxy-D-*glycero*-hex-1-en-3-ulose); cv., cultivar; FW, fresh weight; KA, kojic acid; PPO, polyphenol oxidase.

ACKNOWLEDGMENT

We are thankful for the technical assistance of Rene Brylle Svensson.

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Received for review June 27, 2005. Revised manuscript received September 28, 2005. Accepted October 6, 2005. This work was partially supported by the European Union under the Cell Factory of 5th Framework Program, QLK3-2001-02400.

JF0515274