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Enzyme-Catalyzed Change of Antioxidants Content and Antioxidant Activity of Asparagus Juice

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A pectolytic enzyme preparation from *Aspergillus niger* (pectinase AN) decreased most rutin content and antioxidant activity of asparagus juice. To investigate the mechanism of such loss, we analyzed several possible related enzyme activities in pectinase AN. We found that the activity of pectinase AN to oxidize guaiacol had no significant difference with or without the presence of H_2O_2; thus it was laccase activity, not peroxidase (PO) activity, that pectinase AN contained. We did not find any polyphenol oxidase (PPO) activity in pectinase AN. Laccase in pectinase AN could be the major cause of loss of rutin and antioxidant activity of asparagus juice. When most laccase activity of pectinase AN was inactivated after heating at 70 °C for 1.5 min and incubated with asparagus juice, the loss rate of rutin was only 9% of that treated with unheated pectinase AN, and the antioxidant activity was even increased. Rhamnosidase activity was detected in pectinase AN and can change rutin in asparagus juice to quercetin-3-glucoside, which has higher antioxidant activity than rutin. This may explain the increase of antioxidant activity of asparagus juice treated with heated pectinase AN that still contained some rhamnosidase activity. The discovery of our research is helpful to produce juice with high antioxidant activity and high health benefits in the juice industry.

KEYWORDS: Pectinase from *Aspergillus niger*; rutin; quercetin-3-glucoside; laccase; rhamnosidase; asparagus juice; antioxidant activity

INTRODUCTION

Asparagus (*Asparagus officinalis* L.) is a green vegetable with high health benefits. Asparagus contains a high amount of rutin, glutathione, and other antioxidants (*1*−*3*), which can prevent some cancer, cardio-, and cerebrovascular diseases (*4*). It has been reported that rutin was the major antioxidant of asparagus (*3*, *5*), and antioxidant activity of asparagus was one of the highest among the commonly consumed vegetables (*5*, *6*). Yet, fresh asparagus lignifies and deteriorates quickly after harvest; therefore, asparagus juice can be an alternative product to allow delivery of the healthy components of asparagus.

Pectinase AN is a commercial enzyme used in the juice industry to degrade pectin of plant cell walls and increase the juice yield (*7*). Pectinase AN is a crude enzyme produced by a selected strain of *Aspergillus niger*. The major enzymes of pectinase AN were pectintranseliminase, polygalacturonase, and pectinesterase, and the minor enzymes of pectinase AN include hemicellulase and cellulase, etc. We have studied the polygalacturonase (PG) activity of pectinase AN and its ability to increase the yield of asparagus juice (*8*, *9*). It has been reported that *Aspergillus* strains can produce peroxidase (PO), rutinase, quercetinase (*10*, *11*), and rhamnosidase (*12*, *13*). These enzymes are produced under sub-optimal growth conditions of *Aspergillus* and enable *Aspergillus* to use alternative sources of nutrients in response to nutrient deprivation (*14*). In a previous study, we found that pectinase AN decreased most rutin and antioxidant activity of asparagus juice (*9*), and thus destroyed the health benefit of asparagus juice. The mechanism of such loss has not been reported. The possible enzymes that can oxidize rutin include polyphenol oxidase (PPO), PO, and laccase. PPO catalyzes the *o*-hydroxylation of monophenols to *o*-diphenols and oxidation of *o*-diphenols to *o*-quinones. PO catalyzes the transfer of hydrogen atoms from phenols to hydrogen peroxide (*15*). Laccase can oxidize inorganic and aromatic compounds, especially phenols, with the reduction of O_2 to water at the same time (*16*). The objective of our present study was to investigate the enzyme activities of pectinase AN that caused the total loss of rutin and antioxidant activity of asparagus juice.

MATERIALS AND METHODS

Chemicals. Rutin, quercetin, quercetin-3-glucoside, 2,2′-diphenyl-1-picrylhydrazyl (DPPH), 6-hydroxy-2,5,7,8-tetramethychroman-2-carboxylic acid (Trolox), *p*-nitrophenyl-α-L-rhamnopyranoside, hydrogen peroxide, catechol, dl-3,4-dihydroxyphenylalanine (DL-DOPA), and guaiacol were analytic grade and purchased from Sigma-Aldrich (St. Louis, MO). Methanol and acetic acid were HPLC grade and purchased from Fisher Scientific (Springfield, NJ). A pectinase from *Aspergillus niger* (catalog number P2736 and lot number 110K1348) was purchased from Sigma-Aldrich.

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Analysis of Pectinase AN's Activity To Oxidize Guaiacol (PO and Laccase Activity). PO activity of pectinase AN was determined as follows. Buffer-substrate solution was prepared by mixing 2.5 mL of 3% H_2O_2, 0.5 mL of guaiacol, and 22 mL of 0.1 M sodium acetate−acetic acid buffer at pH 5.8 (*17*, *18*). Pectinase AN (0.1 mL) was added to 1 mL of 25 °C buffer−substrate solution, and the absorbance was recorded at 470 nm for 10 min using an Ultrospec 4000 UV/visible spectrophotometer (Pharmacia Biotech, Cambridge, England). The initial slope of the absorbance curve was used to calculate the PO activity of pectinase AN. The laccase activity of pectinase AN was determined as follows: the buffer−substrate solution was prepared by mixing 2.5 mL of water, 0.5 mL of guaiacol, and 22 mL of 0.01 M sodium phosphate at pH 5.8, and the rest of the steps of the analysis were the same as those for PO activity. One unit of enzyme activity was defined as a change of absorbance of 0.001 per min. The enzyme activity was expressed as Unit/mL pectinase AN.

Analysis of PPO Activity of Pectinase AN. The PPO activity of pectinase AN was determined using either catechol or DL-DOPA as a substrate (*19*, *20*). Pectinase AN (0.1 mL) and 1 mL of 0.01 M sodium phosphate buffer (pH 5.8) were incubated at 30 °C for 5 min, then added with 0.2 mL of 0.5 M catechol to initiate the reaction. The absorbance of the solution was measured at 420 nm for 10 min using a spectrophotometer. PPO activity of pectinase AN was also analyzed using dl-DOPA as a substrate. The mixture of 0.45 mL of 0.05 M acetate buffer (pH 5.8) and 1 mL of 18 mM DL-DOPA was incubated at room temperature for 10 min, and added with 0.05 mL of pectinase AN. The absorbance of the solution was measured at 475 nm for 10 min. A 0.001 change of absorbance per min was defined as one unit of PPO activity. PPO activity of pectinase AN was expressed as Unit/mL enzyme.

Preparation of Asparagus Juice. Green asparagus (*Asparagus officinalis* L. var. Jersey Giant) was harvested from a local farm and immediately shipped to the lab on ice. With the basal part cut off, asparagus spears were 5 in. in length, and were blanched in hot water at 90 °C for 2 min to inactivate the enzymes and frozen at −20 °C. Upon producing asparagus juice, frozen asparagus was thawed, trimmed into small pieces, and macerated in a domestic juicer. The crude juice was centrifuged at 26 712*g* at 4 °C for 15 min in a Beckman J2-HS centrifuge (Beckman, Palo Alto, CA) to obtain the clear juice.

Asparagus Juice Incubated with Heated Pectinase AN. Pectinase AN (1 mL) was heated at 70 °C for 1.5 min in a 10 mL glass culture tube (13 × 100 mm) in a water bath (Precision Scientific Co., Chicago, IL) to inactivate the laccase activity. After being cooled immediately in ice water, pectinase AN solution was centrifuged at 11 872*g* for 10 min, and the laccase activity of the supernatant was analyzed immediately. Next, 40 μL of the supernatant of heated pectinase AN was immediately incubated with 4 mL of asparagus juice (pH 5.8) at 37 °C in a water-bath shaker shaken at 100 rpm. Asparagus juice (0.2 mL) was sampled at 0.5, 1, 2, 4, and 8 h and added with 0.8 mL of methanol to inactivate pectinase AN. The solution was then centrifuged at 11 872*g* for 10 min to get the clear juice to analyze its rutin content and antioxidant activity. Controls were asparagus juice incubated at the same conditions with unheated pectinase AN or without adding any enzyme. The loss rate of rutin or antioxidant activity of asparagus juice was calculated by the slope of the linear regression curve of rutin content or antioxidant activity versus time.

Determination of Antioxidant Activity of Asparagus Juice by the DPPH Method. In a previous study, we used 2,2′-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) and the DPPH method to determine the antioxidant activity of pectinase AN-treated asparagus juice (*9*), which got similar results. Thus, only the DPPH method was used in this research. The DPPH method actually determines the free radical scavenging activity of compounds. Asparagus juice (10, 20, and 40 μL) was added to 1 mL of 0.75×10^{-4} M DPPH methanol solution, respectively, and incubated at room temperature for 30 min (*21*). The absorbance of the DPPH solution was determined at 515 nm using a spectrophotometer. The inhibition percentage of the DPPH solution was calculated according to the following equation:

$$\text{inhibition\%} = (\text{Abs}_{t=0} - \text{Abs}_{t=30\text{min}})/\text{Abs}_{t=0} \times 100$$

$\text{Abs}_{t=0}$ was the absorbance of DPPH solution at time zero, and $\text{Abs}_{t=30\text{min}}$ was the absorbance of DPPH solution after 30 min.

The inhibition% was plotted versus volumes of asparagus juice to get a linear regression curve. The ratio between the slopes of linear regression line of asparagus juice and Trolox was defined as Trolox equivalent antioxidant activity. The antioxidant activity was expressed as mmol Trolox equivalent/L juice.

HPLC Analysis of Rutin and Quercetin-3-glucoside of Asparagus Juice. We used an Agilent 1100 HPLC (Palo Alto, CA) to determine the rutin content of asparagus juice. The HPLC included a quaternary pump, vacuum degasser, thermostatic column compartment, and a diode array detector. The separation was performed on a Vydac 201TP (50 mm × 4.6 mm i.d., 5 μm particle size) guard column and an Agilent Eclipse XDB-C8 column (150 mm × 4.6 mm i.d., 5 μm particle size). Mobile phase A was 5% (v/v) methanol in water with pH adjusted to 2.6 by acetic acid, and mobile phase B was 60% (v/v) methanol in water. Using a gradient elution, mobile phase B was increased from 0 to 50% in 15 min and held at 50% for 8 min. The injection volume was 10 μL, and the flow rate was 0.8 mL/min. Rutin content was quantified at 360 nm using an external standard (0.01−1 mg/mL in methanol). The newly produced compound in asparagus juice treated with heated pectinase AN, which could be quercetin-3-glucoside, was determined by HPLC using the same conditions as those for rutin analysis.

Analysis of α-L-Rhamnosidase Activity of Pectinase AN. Rhamnosidase can cleave the rhamnose residue of rutin to form quercetin-3-glucoside (*13*, *22*). To determine the possible rhamnosidase activity of pectinase AN, 5 μL of pectinase AN, 5 μL of 70 mM *p*-nitrophenyl-α-L-rhamnopyranoside, and 890 μL of 10 mM Tris-HCl buffer (pH 7.8) were mixed in a 2 mL glass vial and incubated at 37 °C for 10 min in a water bath (*23*, *24*). One hundred microliters of 100 mM NaOH solution was added to stop the reaction, and the absorbance of the solution was determined at 420 nm using a spectrophotometer. The molar absorption coefficient of *p*-nitrophenol at 420 nm is 16.0×10^3 M^{-1} cm^{-1}. One unit of rhamnosidase activity was defined as the amount of enzyme to release 1 μmol of *p*-nitrophenol in 1 min. The rhamnosidase activity was expressed as Unit/mL pectinase AN.

Identification of Compounds with Free Radical Scavenging Activity in Asparagus Juice. Asparagus juice (1 mL) treated with heated pectinase AN was mixed with 1 mL of 5 mM DPPH methanol solution and held at room temperature for 3 min. Next, the solution was centrifuged at 11 872*g* for 5 min to remove the undissolved particles. The supernatant was filtered through a 0.45 μm PTFE syringe filter (VWR, So. Plainfield, NJ) and was injected into HPLC for analysis. The compounds with decreased peak areas after adding DPPH reagent had free radical scavenging activity (*25*).

Statistical Analysis. All experiments were performed in triplicate. The mean values and standard deviations were calculated using Excel (Microsoft Inc., Redmond, WA). SYSTAT was used to perform the statistical analysis (Systat Software Inc., Point Richmond, CA). One-way ANOVA and multiple comparisons (Fisher's least-significant-difference test) were used to evaluate the significant differences of the data at the criterion of $\alpha < 0.05$ (*26*).

RESULTS AND DISCUSSION

Mechanism of Pectinase AN in Decreasing Rutin and Antioxidant Activity of Asparagus Juice. We did not detect any PPO activity of pectinase AN because pectinase AN cannot oxidize either catechol or DL-DOPA as determined by the spectral absorbance. We found that pectinase AN oxidized guaiacol, but there was no significant difference in the pectinase AN's activity to oxidize guaiacol with or without the presence of H_2O_2. To catalyze the oxidation of guaiacol, the enzyme needs to transfer the electron from guaiacol to another compound, and this compound was H_2O_2 for PO. Yet, laccase does not need the presence of H_2O_2, and it was O_2 in the air that can be reduced by laccase to water. Therefore, the detected PO activity to oxidize guaiacol was not the real PO activity but laccase activity. Laccase in pectinase AN may decrease most

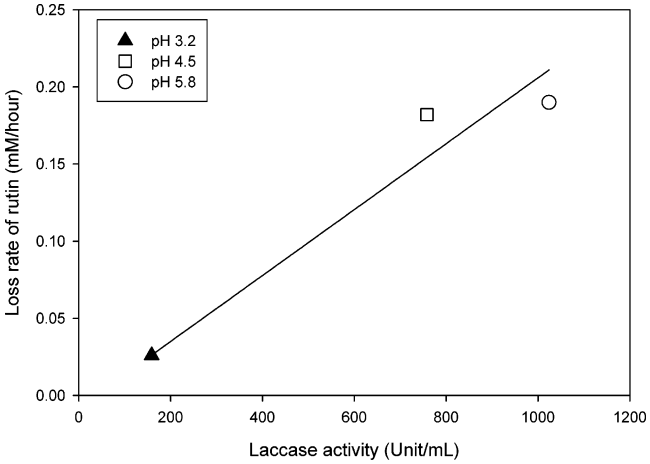

Figure 1. Loss rate of rutin of asparagus juice and the laccase activity of pectinase AN at pH 3.2, 4.5, and 5.8 ($Y = 0.0002*X + 0.0019$, X is the laccase activity of pectinase AN, Y is the loss rate of rutin, $N = 3$, $R = 0.97$, $P < 0.05$). Data are mean ± standard deviation (SD) ($n = 3$); note that the standard deviation bar is too small to show.

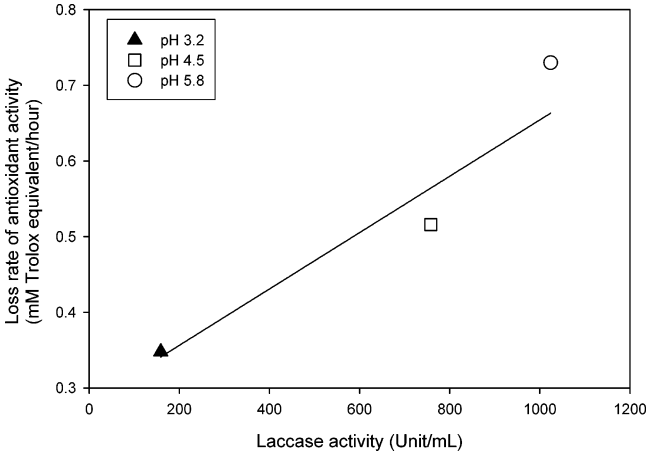

Figure 2. Loss rate of antioxidant activity of asparagus juice and the laccase activity of pectinase AN at pH 3.2, 4.5, and 5.8 ($Y = 0.0004*X + 0.263$, X is the laccase activity, Y is the loss rate of antioxidant activity, $N = 3$, $R = 0.956$, $P < 0.05$). Data are mean ± standard deviation (SD) ($n = 3$); note that the standard deviation bar is too small to show.

rutin and antioxidant activity of asparagus juice. It was reported that laccase can oxidize catechol (*27*, *28*). Yet, we did not found it in this research. The reason could be that (1) different detection methods may have different detection limits; we used spectral absorbance to determine oxidation of catechol by laccase, but in some previous research the analysis was performed by monitoring oxygen uptake using an electrode (*28*); or (2) laccase from different biological sources may have a different activity for catechol.

Pectinase AN's laccase activity at pH 3.2 and 4.5 without adding H_2O_2 was also determined. The laccase activity of pectinase AN varied with the pH's of the analysis buffer, and the order of laccase activity of pectinase AN was pH 3.2 < pH 4.5 < pH 5.8. pH can affect laccase activity of pectinase AN because pH causes different folding and unfolding structures and ionization of prototropic groups in the active site (*29*). When the pH of asparagus juice was adjusted to 3.2 or 4.5 and incubated with unheated pectinase AN, the loss rate of rutin at pH 3.2 and 4.5 was 13.7% and 94.6%, respectively, of that at pH 5.8; the loss rate of antioxidant activity of asparagus juice at pH 3.2 and 4.5 was 47.6% and 70.8%, respectively, of that at pH 5.8. The loss rate of rutin, as well as the loss rate of antioxidant activity, of asparagus juice at pH 3.2, 4.5, and 5.8 showed significant correlation with the laccase activity of pectinase AN at these pH's (**Figures 1** and **2**). In contrast, rutin content and antioxidant activity of asparagus juice without pectinase AN did not change significantly. This confirmed that laccase of pectinase AN could be the major cause of the total loss of rutin and antioxidant activity of asparagus juice.

Pectinase AN was heated at 90 °C for 5 min to completely inactivate its laccase activity, and the rutin content and antioxidant activity of asparagus juice treated with pectinase AN were not decreased (*8*). This showed that the total loss of rutin and antioxidant activity of asparagus juice was not caused by the metal ions (Cu(II), Fe(II), and Fe(III)) that may exist in pectinase AN solution (*30*), but the enzymes of pectinase AN.

Rutin Content and Antioxidant Activity of Asparagus Juice Treated with Heated Pectinase AN. Laccase activity of pectinase AN was decreased from 646 to 4 Unit/mL after heating at 70 °C for 1.5 min. Because heated pectinase AN had little remaining laccase activity, the loss rate of rutin of the treated asparagus juice (0.04 mM/h) was only 9% of that treated with unheated pectinase AN (0.45 mM/h) (**Figure 3**). The

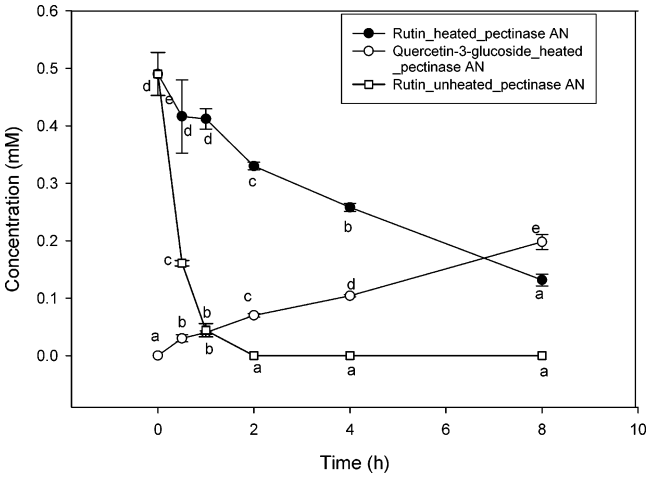

Figure 3. Concentrations of rutin and quercetin-3-glucoside of asparagus juice incubated with unheated and heated pectinase AN at 37 °C. Data are mean ± SD ($n = 3$). Means with different letters in the same curve represent a significant difference ($p < 0.05$).

laccase of pectinase AN seemed less heat stable as compared to that in previous research (*27*), which could be due to different biological sources of laccase. No significant change in rutin content and antioxidant activity of asparagus juice without adding any enzyme was found. The antioxidant activity of asparagus juice treated with heated pectinase AN was significantly increased after 8 h (**Figure 4**), the reason of which was explained as follows.

Quercetin-3-glucoside of Asparagus Juice Treated with Heated Pectinase AN and Rhamnosidase Activity of Pectinase AN. In the HPLC chromatogram of asparagus juice treated with heated pectinase AN, we found a new compound (peak 3) that eluted right after the rutin peak (**Figure 5B**). Peak 3 could be quercetin-3-glucoside because peak 3 had: (1) the same retention time as the spiked quercetin-3-glucoside standard (**Figure 5C**); and (2) UV spectra similar to those of quercetin-3-glucoside standard (*8*, *31*). For asparagus juice treated with heated pectinase AN, the concentration of rutin was decreased and the concentration of quercetin-3-glucoside was increased, correspondingly (**Figure 3**). Antioxidant activity of rutin and quercetin-3-glucoside standard (0.01−1.0 mg/mL in methanol) was analyzed by the DPPH method, which showed that

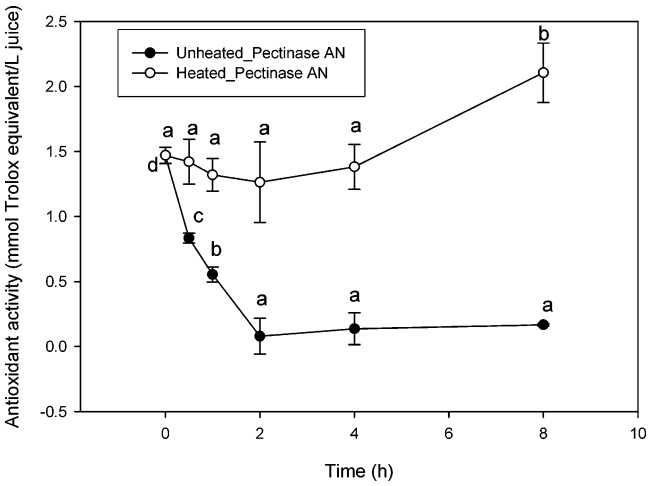

Figure 4. Antioxidant activity of asparagus juice incubated with unheated and heated pectinase AN at 37 °C determined by the DPPH method. Data are means ± SD ($n = 3$). Means with different letters in the same curve represent a significant difference ($p < 0.05$).

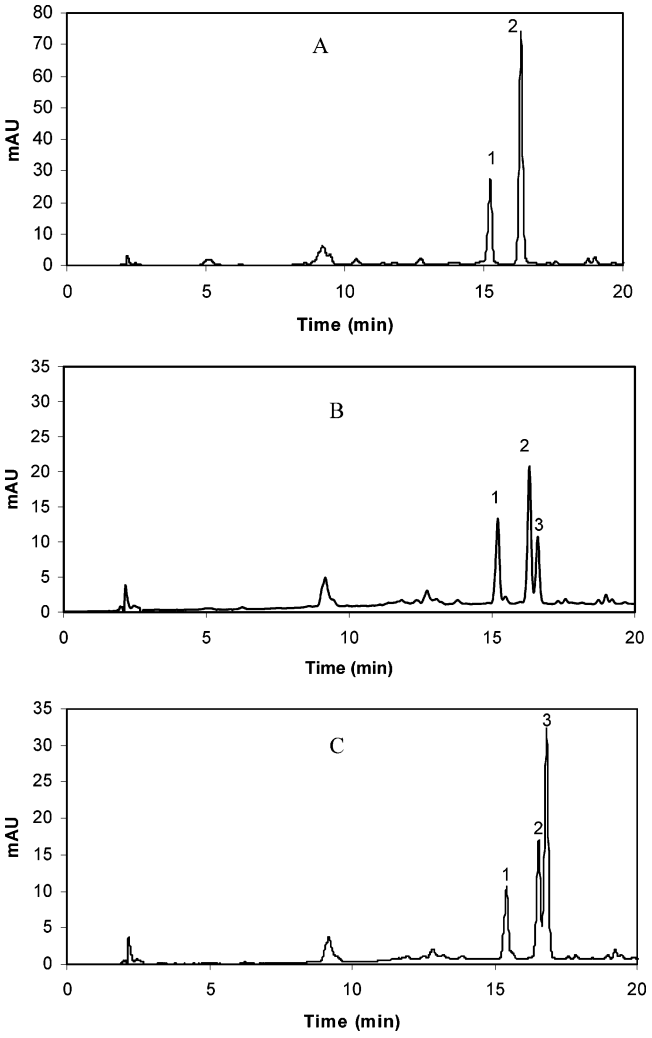

Figure 5. HPLC chromatogram of asparagus juice heated at 37 °C for 4 h and determined by HPLC at 360 nm, (A) without treatment of pectinase AN, (B) treated with heated pectinase AN, and (C) treated with heated pectinase AN and spiked with quercetin-3-glucoside standard (peak 1 was unknown, peak 2 was rutin, and peak 3 was quercetin-3-glucoside).

quercetin-3-glucoside has higher antioxidant activity (2.92 ± 0.20 Trolox equivalent, $n = 3$) than rutin (1.61 ± 0.10 Trolox equivalent, $n = 3$). This result agreed with one study using a different method to assess the antioxidant activity (*32*). This may explain the increase of antioxidant activity of asparagus juice after 8 h of treatment with heated pectinase AN. Quercetin content of asparagus juice treated with heated pectinase AN was not detected. Peak 1 (unknown), peak 2 (rutin), and peak 3 (quercetin-3-glucoside) of asparagus juice all had decreased peak areas after reaction with DPPH free radicals, which showed that compounds of peaks 1−3 are all potential antioxidants.

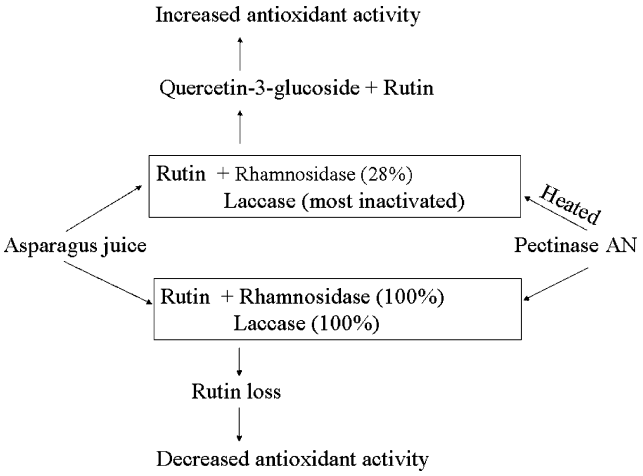

Figure 6. Effects of unheated and heated pectinase AN on rutin content and antioxidant activity of asparagus juice.

From the result, it was suggested that rhamnosidase may exist in pectinase AN as rhamnosidase can change rutin to quercetin-3-glucoside. To our expectation, rhamnosidase activity of pectinase AN was detected, which agreed with previous research that *Aspergillus niger* can produce rhamnosidase (*12*, *33*). Heating at 70 °C for 1.5 min reduced the rhamnosidase activity of pectinase AN from 0.56 to 0.14 Unit/mL. Quercetin-3-glucoside was not detected in asparagus juice treated with unheated pectinase AN because quercetin-3-glucoside could be further oxidized by laccase of pectinase AN.

Above all, the effect of pectinase AN on rutin content and antioxidant activity of asparagus juice can be explained as follows (**Figure 6**). Laccase of pectinase AN oxidized most rutin and other antioxidants of asparagus juice, and the antioxidant activity of asparagus juice was decreased. Heated pectinase AN, with little laccase activity and a little rhamnosidase activity, changed rutin to quercetin-3-glucoside and increased antioxidant activity of asparagus juice. We first reported laccase of pectinase AN as the major cause of total loss of rutin and antioxidant activity of asparagus juice. Pectinase AN is commonly used in the production of fruit and vegetable juices in the food industry. The total loss of antioxidant activity of asparagus juice caused by pectinase AN could occur to any juice, because laccase can oxidize not only rutin but also many other phenolic antioxidants in apple, orange, or grape juices. Therefore, it is necessary to analyze the laccase activity of pectolytic enzymes preparations before using it to produce juice. If the laccase activity of the enzyme is high, this enzyme cannot be directly used to produce juice. The discovery of our research is very important to the juice industry to produce juice with high antioxidant activity.

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