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Loss of rutin and antioxidant activity of asparagus juice caused by a pectolytic enzyme preparation from *Aspergillus niger*

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Abstract

We found that a commercial pectolytic enzyme preparation from *Aspergillus niger* (pectinase AN) contained laccase activity that decreased rutin content and antioxidant activity of asparagus juice. This research investigated the effects of pH, temperature, and concentration of pectinase AN on pectinase AN's laccase activity to decrease rutin content and antioxidant activity of asparagus juice. Asparagus juice was incubated with pectinase AN at different pHs (3.2, 4.5 and 5.8), temperatures (25, 37, and 50 °C) and enzyme concentrations (0.1%, 0.5% and 1%). Rutin content and antioxidant activity of samples was determined by HPLC and 2,2′-diphenyl-1-picrylhydrazyl (DPPH) free radical method, respectively. The rate of loss of rutin and antioxidant activity of asparagus juice was smaller at pH 3.2 than at pH 4.5 and pH 5.8, smaller for 0.1% pectinase AN than 0.5% and 1% pectinase AN. The rate of loss of rutin of asparagus juice was greater at 25 °C than at the other two temperatures. Pectinase AN can decrease rutin content and antioxidant activity of asparagus juice at the selected conditions. But rutin content and antioxidant activity of asparagus juice produced using pectinase AN could be less decreased at pH 3.2 and 0.1% of enzyme with less than 2 h of incubation time. This information was helpful for juice industry to produce juices with high antioxidant activity using pectinase AN.
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Keywords: Pectinase from *Aspergillus niger*; Rutin; Asparagus juice; Antioxidant activity; Laccase

1. Introduction

Asparagus (*Asparagus officinalis*, family Liliaceae) is a green vegetable with high antioxidant activity among the commonly consumed vegetables (Vinson, Hao, Su, & Zubik, 1998). The antioxidants in asparagus include ascorbic acid, rutin, glutathione, etc. (Shao et al., 1997), and rutin has been reported to be the major antioxidant (Tsushida, Suzuki, & Kurogi, 1994). Antioxidants have the function to scavenge free radicals and reduce the oxidative stress in human (Jarrett & Boulton, 2005), which can prevent some cardiovascular diseases, cancers and aging (Mantovani et al., 2003; Willcox, Ash, & Catignani, 2004). As asparagus deteriorate quickly after harvest, it is often processed to extend its shelf life. Asparagus juice could be a potential product for human consumption.

To produce juice, pectolytic enzymes are commonly used to degrade pectins in plant cell walls and increase the yield (Will, Schulz, Ludwig, Otto, & Dietrich, 2002). But these crude enzymes may contain small amount of enzymes that can degrade phenolics. For example, β-glucosidase degraded anthocyanins in strawberry, raspberry and grape juices (Le Traon-Masson & Pellerin, 1998; Versari, Biesenbruch, Barbanti, Farnell, & Galassi, 1997; Wightman & Wrolstad, 1996). In examining several commercial pectolytic enzyme preparations for their ability to cleave the phenolic glycoside of rutin, we found that an enzyme preparation from *Aspergillus niger* (pectinase AN) decreased most rutin content and antioxidant activity of asparagus juice, which could be caused mainly by laccase

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activity of pectinase AN (Sun, Powers, & Tang, 2007; Sun, Tang, & Powers, 2005). We also found pectinase AN contained a small amount of rutinase activity that can change rutin to quercetin, as well as rhamnosidase activity that can change rutin to quercetin-3-glucoside (Sun et al., 2007; Sun et al., 2005). Quercetin was not detected or at very small content in asparagus juice treated with 1% pectinase AN at pH 5.8 and 37 °C (Sun et al., 2005). When laccase activity of pectinase AN was mostly inactivated by heating and a small amount of rhamnosidase activity remained, quercetin-3-glucoside was found in asparagus juice treated with the heated pectinase AN (Sun et al., 2007). However, impact factors, such as pH, temperature, and concentration of pectinase AN, that could affect pectinase AN's activity in decreasing rutin content and antioxidant activity of asparagus juice has not been systematically studied. It is critical to know at what circumstances the loss of rutin and antioxidant activity of asparagus juice was minimized. The objectives of our present research were to investigate the effects of these potential factors on the loss rate of rutin and antioxidant activity asparagus juice and provide scientific information to juice industry.

2. Materials and methods

2.1. Chemicals

Rutin, quercetin, 6-hydroxy-2,5,7,8-tetramethychroman-2-carboxylic acid (Trolox), 2,2′-diphenyl-1-picrylhydrazyl (DPPH) free radical, and guaiacol were analytical grade and purchased from Sigma–Aldrich (St. Louis, MO, USA). Methanol and acetic acid (HPLC grade) were purchased from Fisher Scientific (Springfield, NJ, USA). Pectinase from *Aspergillus niger* was purchased from Sigma–Aldrich (Catalog # P2736, lot # 110K1348).

2.2. Pectinase AN in rutin solution

As asparagus juice is a complex system containing many factors that may affect the activity of pectinase AN, to simply the system, the function of pectinase AN on rutin was investigated in a model system of rutin solution. Rutin was dissolved in 20% (v/v) methanol to form a saturated solution and the pH of the solution was adjusted to 5.8 with sodium acetate. Then the solution was filtered through a Whatman # 42 filter paper to remove the undissolved particles. Rutin solution (4 ml) was incubated with 60 μl pectinase AN in a capped 20 ml glass vial at 37 °C. The solution was sampled at 0, 0.5, 1, 2 and 4 h and three volumes of methanol was added to inactivate pectinase AN. Control was rutin solution incubated without pectinase AN at the same conditions. Rutin content and antioxidant activity of the sample were determined as described later.

Effect of methanol content of solution on laccase activity of pectinase AN was determined as follows: 2.5, 5 and 10 ml of methanol was mixed with 19.5, 17 and 12 ml of 0.1 M sodium acetate-acetic acid buffer (pH 5.8), respectively, which was then added with 0.5 ml guaiacol and 2.5 ml water to obtain the solution with methanol content of 10%, 20% and 40%, respectively. The final volume of the mixed solution was 25 ml. One ml of the solution was added with 0.0625 ml pectinase AN and the absorbance was recorded at 470 nm for 10 min using an Ultrospec 4000 UV/Vis spectrophotometer (Pharmacia Biotech, Cambridge, England). The initial slope of the absorbance curve was used to calculate the laccase activity. One unit of laccase activity was defined as a change of absorbance of 0.001 per min. Laccase activity was expressed as Unit/ml pectinase AN.

2.3. Asparagus Juice incubated with pectinase AN at different conditions

Green asparagus (*Asparagus officinalis* family Liliaceae var. Jersey Giant) was harvested from a local farm. The basal part of asparagus was removed and the remained asparagus spear was 5 in. in length. Asparagus spears were blanched in hot water at 90 °C for 2 min to inactivate the enzymes and stored in plastic bags at −20 °C. Frozen asparagus was thawed, trimmed into small pieces and macerated in a domestic juicer (Mod. 1738 Braun, Germany) to produce juice. Crude juice was centrifuged at 26,712*g* for 15 min at 4 °C and the clear juice was collected for further experiment.

Asparagus juice (4 ml) was incubated with pectinase AN at different conditions (pH, temperature and concentrations of pectinase AN): (1) Asparagus juice was adjusted to pH 3.2, 4.5 and 5.8 using 2.8 M citric acid, respectively, which was then incubated with 1% (v/v) pectinase AN at 37 °C; (2) 0.1%, 0.5% and 1% (v/v) of pectinase AN was incubated with asparagus juice (pH 5.8) at 37 °C, respectively; (3) Asparagus juice was incubated with 1% (v/v) pectinase AN at pH 5.8 at 25, 37 and 50 °C, respectively. Asparagus juice was sampled at 0, 0.5, 1, 2, 4 and 8 h, respectively, and four volumes of methanol were added to asparagus juice to inactivate pectinase AN. Control was asparagus juice incubated without pectinase AN at the same conditions (pH 5.8 and temperature 37 °C). Rutin content and antioxidant activity of asparagus juice were determined as described later.

Laccase activity of pectinase AN at different pHs was determined. Buffer with pH 3.2, 4.0, 4.5, 5.0, 5.8 and 6.2 was prepared by mixing different ratios of 0.1 M sodium acetate and 0.1 M acetic acid solution, respectively; buffer with pH 6.5, 7.0, 7.5 and 8.0 was prepared by mixing different ratios of 0.1 M dibasic sodium phosphate and 0.1 M sodium hydrogen phosphate solution, respectively (Stoll & Blanchard, 1990). Then 22 ml buffer of a certain pH, 2.5 ml H_2O, and 0.5 ml guaiacol was mixed, 1 ml of which was incubated with 0.0625 ml pectinase AN. The absorbance of the solution was recorded at 470 nm for 10 min using a spectrophotometer to determine the laccase activity of pectinase AN.

2.4. HPLC analysis of rutin, quercetin and quercetin-3-glucoside

The Agilent 1100 HPLC system used for analysis included a quaternary pump, vacuum degasser, thermostatic column compartment and a diode array detector (Palo Alto, CA). The separations were performed on an Agilent Eclipse XDB-C8 column (150 mm × 4.6 mm i.d., 5 μm particle size) with a Vydac 201TP (Columbia, MD) guard column at 40 °C. The mobile phase was acetic acid, methanol and water (5:40:55%, v/v) at the flow rate of 0.8 ml/min. Injection volume of sample was 10 μl. The wavelength of the detector was set at 360 nm. Rutin, quercetin and quercetin-3-glucoside content of sample was quantified using external standards (0.01–1 mg/ml).

2.5. Determination of antioxidant activity by DPPH free radical method

It has been suggested that at least two methods should be used to measure the antioxidant activity (Prior, Wu, & Schaich, 2005). In a previous study, we have found that DPPH radical and 2,2′-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) radical method showed similar results in ranking the antioxidant activity of asparagus juice (Sun et al., 2005). Therefore, in the present study, only DPPH free radical method was used to determine the antioxidant activity (Brand-Williams, Cuvelier, & Berset, 1995; Sun et al., 2005). Aliquots of sample was added to 1 ml DPPH free radical (0.75×10^{-4} M) ethanol solution and kept in the dark at 25 °C for 30 min. The absorbance of DPPH free radical was determined at 515 nm using a spectrophotometer. The percentage of reduction of the absorbance (inhibition%) due to quenching the DPPH radical was calculated using the following equation:

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

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

The inhibition% was plotted versus the amount of sample to obtain a linear regression curve. The slope of such a curve of a sample was compared to that of Trolox, and the ratio between the two slopes was defined as the Trolox equivalent antioxidant activity (TEAC).

2.6. Statistical analysis

The experiments were performed in triplicates. One-way ANOVA and multiple comparisons (Fisher's least-significant-difference test) were used to evaluate the differences among treatments, and linear and nonlinear regressions were performed to see the trend generated from the experiment (Zar, 1996). For all the statistics, $\alpha = 0.05$ and 0.01 were set as the significant and highly significant criteria. SYSTAT was used to perform the statistical analysis (Systat Software Inc., Point Richmond, CA).

3. Results and discussion

3.1. Rutin content and antioxidant activity of rutin solution treated with pectinase AN

Rutin content of rutin solution without pectinase AN did not change significantly (Fig. 1). But, for rutin solution treated with pectinase AN, rutin content was significantly decreased, and quercetin and quercetin-3-glucoside was also detected. Quercetin content was increased, but quercetin-3-glucoside content first increased then decreased. The total content of the three compounds was not constant but significantly decreased slightly between 4 and 13 h (Fig. 1). The antioxidant activity of rutin solution with and without pectinase AN was significantly decreased a little between 0 and 2 h and did not change significantly between 2 and 13 h, but the value of the former was a little significantly lower than the latter between 1 and 4 h (Fig. 2).

This result was different from our previous result that rutin in asparagus juice was totally oxidized by pectinase AN without any quercetin and quercetin-3-glucoside produced (Sun et al., 2005), because laccase activity of pectinase AN in 20% methanol was only 9.6% of its activity in water and cannot degrade as much rutin as in asparagus juice (0% methanol) (laccase activity of pectinase AN decreased with the increased methanol content and was 23.1% and 9.5% in 10% and 40% of methanol, respectively, compared to that in water). At the same time, pectinase AN contained both rutinase and rhamnosidase activity (Sun et al., 2007; Sun et al., 2005) and rutinase activity in 20% methanol was reported to be even greater than in water (Yasuda & Shinoyama, 1995), thus, quercetin-3-glucoside and significant amount of quercetin was produced in rutin solution. The produced quercetin in rutin solution may not be oxidized by laccase of pectinase AN, as we

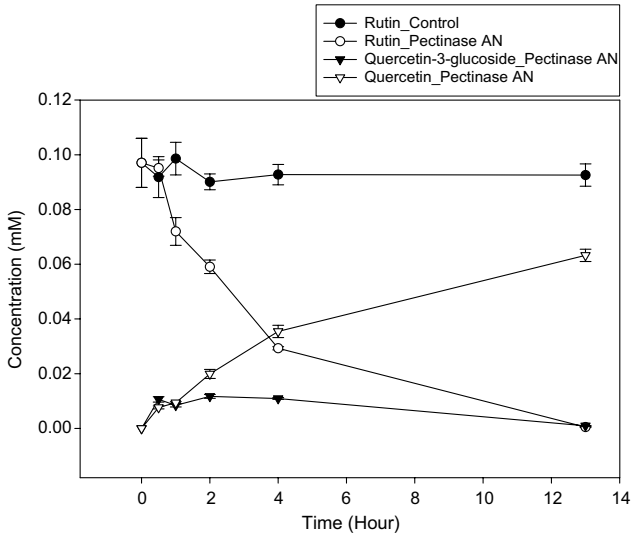

Fig. 1. Rutin, quercetin and quercetin-3-glucoside content of rutin solution incubated with 1.5% (v/v) pectinase AN at pH 5.8 at 37 °C. Data are expressed as means ± SD ($n = 3$).

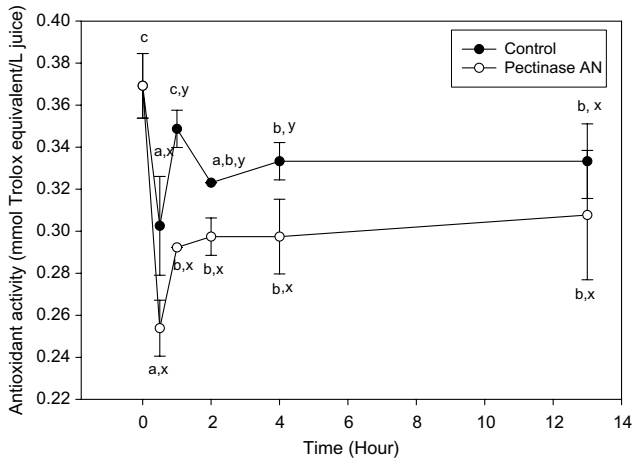

Fig. 2. Antioxidant activity of rutin solution incubated with 1.5% (v/v) pectinase AN at pH 5.8 at 37 °C determined by DPPH free radical method. Data are expressed as means ± SD ($n = 3$). Means with different letters (a–c) or (x, y) represent a significant difference with time or with pectinase AN treatment, respectively ($p < 0.05$).

found that quercetin content did not change significantly in quercetin solution (quercetin dissolved in 20% methanol) treated with pectinase AN. Some researchers reported that quercetin was not oxidized by laccase (Gomes, Nogueira, & Rebelo, 2004; Gomes & Rebelo, 2003) and some reported that quercetin could be oxidized by laccase (Desentis-Mendoza et al., 2006; Pereira, Bastos, Tzanov, Cavaco-Paulo, & Guebitz, 2005). The reason could be that laccases from different sources at different environmental conditions (pH, temperature, and ionic strength, etc.) could show different activity toward quercetin.

3.2. Rutin content and antioxidant activity of asparagus juice treated with pectinase AN

Both rutin and antioxidant activity of asparagus juice was significantly decreased and their loss rate was significantly affected by pH, temperature and concentration of pectinase AN, except for antioxidant activity of asparagus juice at different temperatures. Rutin content and antioxidant activity of asparagus juice without adding pectinase AN did not change significantly. The loss rate of rutin or antioxidant activity of asparagus juice was calculated by the slope of the regression curve of rutin content or antioxidant activity versus time (only data in the linear range was used). The loss rate of rutin of asparagus juice at pH 3.2 and 4.5 was 13.7% and 94.6%, respectively, of that at pH 5.8 (Table 1), which was in agreement with the rank of laccase activity of pectinase AN at different pHs, which was pH 3.2 < H 4.5 < H 5.8. The relationship between pH and laccase activity of pectinase AN was: $Y = -82.34 * X^2 + 999.83 * X - 2086.9$, $N = 10$, $R = 0.80$, $P < 0.01$, Y is the laccase activity and X is the pH value. The highest laccase activity of pectinase AN was found between pH 5.5 and 6.5 (Fig. 3), which was similar to that the maximum laccase activity from *Coriolus versicolor* was observed between 4.5 and 5.5 (Gomes & Rebelo,

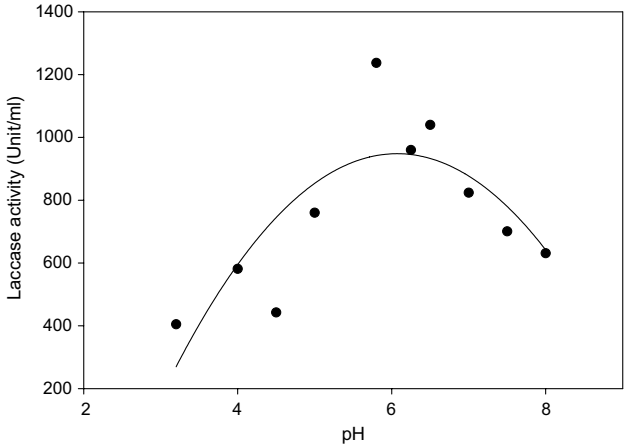

Fig. 3. Laccase activity of pectinase AN in aqueous solution at selected pHs at 25 °C. The data is the average of three replicates.

Table 1
Rutin content (mM) of asparagus juice incubated with pectinase AN at different conditions (mean ± SD, $n = 3$)

Conditions	0 h	0.5 h	1.0 h	2.0 h	4.0 h	8.0 h
pH						
3.2	0.41 ± 0.00	0.41 ± 0.02	0.38 ± 0.01	0.36 ± 0.01	0.29 ± 0.00	0.21 ± 0.00
3.2[a]	0.00 ± 0.00	0.02 ± 0.00	0.02 ± 0.00	0.03 ± 0.00	0.02 ± 0.00	0.01 ± 0.00
4.5	0.41 ± 0.00	0.38 ± 0.01	0.26 ± 0.01	0.06 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
5.8	0.37 ± 0.00	0.33 ± 0.00	0.15 ± 0.01	0.01 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
Temperature (°C)						
25	0.41 ± 0.00	0.19 ± 0.02	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
37	0.37 ± 0.00	0.33 ± 0.00	0.15 ± 0.01	0.01 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
50	0.41 ± 0.00	0.24 ± 0.03	0.08 ± 0.03	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
Pectinase AN (%)						
0.10	0.41 ± 0.00	0.42 ± 0.05	0.40 ± 0.02	0.36 ± 0.01	0.23 ± 0.01	0.09 ± 0.03
0.50	0.41 ± 0.01	0.33 ± 0.01	0.20 ± 0.01	0.03 ± 0.01	0.00 ± 0.00	0.00 ± 0.00
1.00	0.37 ± 0.00	0.33 ± 0.00	0.15 ± 0.01	0.01 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
Control	0.37 ± 0.00	0.38 ± 0.01	0.36 ± 0.01	0.36 ± 0.01	0.39 ± 0.01	0.38 ± 0.00

[a] Quercetin content (mM) at pH 3.2.

Table 2
Antioxidant activity of asparagus juice (mmol Trolox equivalent/L juice) incubated with pectinase AN at different conditions (mean ± SD, $n = 3$)

Conditions	0 h	0.5 h	1.0 h	2.0 h	4.0 h	8.0 h
pH						
3.2	1.39 ± 0.05	1.20 ± 0.11	0.95 ± 0.24	0.70 ± 0.09	0.74 ± 0.03	0.68 ± 0.03
4.5	1.39 ± 0.05	0.79 ± 0.28	0.64 ± 0.03	0.27 ± 0.01	0.09 ± 0.03	0.11 ± 0.06
5.8	1.70 ± 0.15	1.36 ± 0.04	0.75 ± 0.08	0.27 ± 0.01	0.14 ± 0.00	0.20 ± 0.02
Temperature (°C)						
25	1.39 ± 0.05	0.93 ± 0.03	0.36 ± 0.03	0.25 ± 0.04	0.25 ± 0.06	0.28 ± 0.06
37	1.70 ± 0.15	1.36 ± 0.04	0.75 ± 0.08	0.27 ± 0.01	0.14 ± 0.00	0.20 ± 0.02
50	1.39 ± 0.05	0.93 ± 0.14	0.54 ± 0.17	0.18 ± 0.03	0.16 ± 0.00	0.17 ± 0.00
Pectinase AN (%)						
0.10	1.39 ± 0.05	1.32 ± 0.13	1.13 ± 0.03	1.07 ± 0.10	0.91 ± 0.00	0.63 ± 0.20
0.50	1.39 ± 0.05	1.14 ± 0.03	0.78 ± 0.02	0.33 ± 0.01	0.18 ± 0.07	0.23 ± 0.07
1.00	1.70 ± 0.15	1.36 ± 0.04	0.75 ± 0.08	0.27 ± 0.01	0.14 ± 0.00	0.20 ± 0.02
Control	1.70 ± 0.15	1.75 ± 0.15	1.70 ± 0.02	1.92 ± 0.04	1.68 ± 0.09	1.58 ± 0.04

2003). The loss rate of rutin in asparagus juice at pH 3.2, 4.5 and 5.8 showed a significant correlation with laccase activity of pectinase AN at these pHs ($Y = 0.0002 * X + 0.0019$, X is the laccase activity, Y is the loss rate of rutin, $N = 3$, $R = 0.97$, $P < 0.05$). Quercetin was detected in asparagus juice treated with pectinase AN at pH 3.2 (Table 1), possibly due to the reduced laccase activity at pH 3.2 that less rutin was oxidized and more rutin was changed to quercetin by rutinase. The loss rate of rutin in the first 2 h was significantly greater at 25 °C than at 37 and 50 °C (Table 1). The rate of loss of rutin in asparagus juice incubated with 0.5% and 1% (v/v) of pectinase AN was similar, and was 4.3 times greater than that with 0.1% pectinase AN (Table 1).

The rate of loss of antioxidant activity of asparagus juice: (1) was 47.6% at pH 3.2 and 70.8% at pH 4.5, respectively, of that at pH 5.8 (Table 2); (2) did not differ significantly at 25, 37 and 50 °C (Table 2); (3) was 15.9% for 0.1% pectinase AN and 73.6% for 0.5% pectinase AN, respectively, compared to that for 1% of pectinase AN (Table 2). Both rutin content and antioxidant activity of asparagus juice treated with pectinase AN were significantly greater at pH 3.2 than that at pH 4.5 and 5.8 (Tables 1 and 2). The loss rate of rutin showed a significant positive relationship with the loss rate of antioxidant activity of asparagus juice ($Y = 0.18 * \mathrm{Ln}(X) + 0.89$, X: loss rate of rutin, Y: loss rate of antioxidant activity, $R = 0.76$, $N = 9$, $P < 0.01$). This correlation showed that rutin was a major antioxidant of asparagus juice (Tsushida et al., 1994).

4. Summary

Our results showed that laccase activity of pectinase AN was smaller in 20% methanol than in water, and the percentage of loss of rutin and antioxidant activity of rutin solution was smaller compared to that in asparagus juice. The rate of loss of rutin and antioxidant activity of asparagus juice was significantly affected by pH, temperature and concentration of pectinase AN. Rate of loss was smaller at pH 3.2 than at pH 4.5 and 5.8, and smaller for 0.1% than 0.5 and 1% pectinase AN. Laccase in pectinase AN can oxidize not only rutin but also many other phenolic antioxidants in apple, orange or grape juices. As the loss of rutin and antioxidant activity of asparagus juice cannot be avoided at the selected conditions caused by pectinase AN, it is suggested that pectinase AN be used at pH 3.2 and 0.1% of concentration with shorter incubation time to prevent its negative effect on rutin and other antioxidants in asparagus juice and other juices. Our results could help juice industry to produce juices with high antioxidant activity.

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