Degradation Kinetics of Catechin in Aqueous Solution in the Presence of Ascorbic Acid or Octanoyl Ascorbate

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The amphiphilic antioxidant, such as acyl ascorbate, would be expected to be a useful food additive. In this study, the antioxidative action of acyl ascorbate was examined, because knowledge of its behavior in aqueous solution is required for its effective use for food. First, the degradation kinetics of (+)-catechin in aqueous solution at various pHs and temperatures were empirically expressed by the Weibull equation. The rate constant, k , was the lower at the lower pH and temperature. The temperature-dependency of the k value was analyzed on the basis of the Arrhenius equation, and it was indicated that the enthalpy-entropy compensation held during the degradation. Second, octanoyl L-ascorbate was synthesized by the condensation of L-ascorbic and octanoic acids in acetone using immobilized lipase from *Candida antarctica*, and the degradation kinetics of catechin in aqueous solution in the presence of octanoyl ascorbate was examined. The dependency of the k value in the catechin solution with octanoyl ascorbate on the concentration of the ascorbate was obviously different from that with ascorbic acid. That is, the degradation was suppressed at low octanoyl ascorbate concentrations, while proceeded fast at a high concentration, suggesting the formation of the micelle for octanoyl ascorbate at high concentrations.

Key words: Catechin, degradation kinetics, ascorbic acid, octanoyl ascorbate, micelle formation

1. Introduction

L-Ascorbic acid is a water-soluble vitamin known as vitamin C, and widely used as an additive in foods and cosmetics due to its strong reducing ability. The lipase-catalyzed synthesis of 6-*O*-acyl ascorbate through the condensation of ascorbic acid and fatty acid in an organic solvent has been reported [1,2]. The enzymatic synthesis of the acyl ascorbates would be more advantageous than a chemical method due to the simplicity of its reaction process and its high regioselectivity. Acyl ascorbate is an amphiphilic antioxidant, as it consists of ascorbic acid and a fatty acid as the hydrophilic antioxidant and lipophilic group, respectively. It has been reported that the ascorbates, such as 6-*O*-palmitoyl ascorbate, have an antitumor activity and metastasis-inhibitory effects [3,4]. Therefore, acyl ascorbate would be expected to be a use-

ful food additive. We have also synthesized acyl ascorbates using an immobilized lipase in a batch or continuous reactor [5,6], evaluated their surfactant properties [7] and their antioxidative ability against linoleic acid [8], and used them for the microencapsulation of lipids [9]. However, there has been no report about the antioxidative property of acyl ascorbate against a water-soluble and unstable compound in aqueous solution, though knowledge of its behavior in aqueous solution is required for its effective use.

Catechins are recognized as constituents of green tea with an antioxidative activity [10] and suppressive ability against adipocyte differentiation [11]. Most tea drinks containing catechins are low acid beverages, and kept warm in the vending system during the cold season. Furthermore, heat processing at high temperature is required to sterilize the spores of thermophilic anaerobes. Thus, the stability of tea catechins in aqueous solution has been investigated under various conditions by many researchers [12,13], and the degradation is kinetically examined using first-order kinetics [14,15].

In this study, the degradation of (+)-catechin in aqueous solution at various pHs and temperatures were determined. The degradation was expressed by the probabilistic Weibull equation and the kinetic parameters were evaluated. Octanoyl ascorbate, which is a hydrophilic surfactant, was synthesized using an immobilized lipase by the condensation of ascorbic and octanoic acids in acetone. The degradation kinetics of catechin in an aqueous solution in the presence of either ascorbic acid or octanoyl ascorbate was also examined.

2. Materials and Methods

2.1 Materials

(+)-Catechin (purity: 98%) was purchased from Sigma (St. Louis). Caffeine, which was used as the internal standard for the determination of catechin by HPLC, was purchased from Kishida Chemical (Osaka). L(+)-Ascorbic acid and octanoic acid were purchased from Nacalai Tesque (Kyoto), and Wako Pure Chemical Industries (Osaka), respectively. All other chemicals of analytical grade were purchased from Wako and Yoneyama Chemical (Osaka).

Octanoyl L-ascorbate was synthesized by the condensation of ascorbic and octanoic acids in acetone using immobilized lipase, Chirazyme® L-2 C2, from *Candida antarctica* fraction B (Roche Molecular Biochemicals (Mannheim)) and purified according to previous methods [2].

2.2 Degradation of catechin under various conditions

Catechin was dissolved in 0.2 mol/L sodium phosphate buffer, pH 6.5, to produce 10 mmol/L. The catechin solution was diluted with the buffer to prepare the catechin solutions of 1.0 and 2.5 mmol/L. Each solution (10 mL) was put in an amber vial and the vial was kept at 37°C in a thermostated water bath. At appropriate intervals, a portion of the solution (50 µL) was removed from the vial, and mixed with 50 µL of 1.3 mmol/L caffeine and 900 µL of the eluent for HPLC analysis. The mixture (20 µL) was applied to a Chemcosorb300-5C18 column (4.6 mm ϕ × 250 mm; Chemco Scientific, Osaka) and eluted with a mixture of methanol, water and phosphoric acid (20/80/0.5 by vol.) at 1.0 mL/min using a Shimadzu LC-10AT pump (Kyoto). The elution profiles of the catechin and caffeine were monitored using a Shimadzu SPD-10A UV detector at 280 nm.

One mmol/L catechin solutions were prepared by dis-

solving it with 0.2 mol/L sodium phosphate buffers of pH 5.0, 6.0, 6.5, 7.0 and 7.5. These pHs were selected, because most tea drinks containing catechin were low acid beverages. The solutions were kept at 37°C, 50°C, 60°C or 70°C, and the concentrations of the remaining catechin were determined by the above-mentioned procedures. Temperature was, for convenience, selected to obtain the results within short time.

2.3 Degradation of catechin in the presence of ascorbic acid or octanoyl ascorbate

Ascorbic acid or octanoyl ascorbate was dissolved in 0.2 mol/L sodium phosphate buffer, pH 6.0, to obtain each solution at the concentration of 10 mmol/L. Each solution was mixed with 10 mmol/L catechin solution at the appropriate volume. The concentration of ascorbic acid or octanoyl ascorbate was from 0.001 to 5 mmol/L, and that of catechin was 1.0 mmol/L. Ten milliliters of each mixture was put in an amber vial and the vial was immersed in a water bath at 60°C. The catechin solution with octanoyl ascorbate was kept at various temperatures from 37°C to 70°C. The sampling and HPLC analysis were carried out by the above methods.

2.4 Partition coefficient of catechin between methyl octanoate and water

The catechin solution dissolved in 0.2 mol/L sodium phosphate buffer, pH 6.5, was prepared at the concentrations of 0.1, 1 and 10 mmol/L, which were the initial concentrations, $C_{aq, 0}$, in the water phase. Five milliliters of the catechin solution and methyl octanoate as the organic phase were mixed in an amber vial. The vial was immersed in a water bath at 37°C, then vigorously shaken for 1 h. Fifty microliters of the water phase in the mixture was removed, and the concentration of catechin in the water phase at the partition equilibrium, C_{aq} , was measured by the above-mentioned HPLC analysis.

The mass balance for the partition of catechin between methyl octanoate and water is expressed by the following equation:

$$C_{aq, 0} V_{aq} = C_{aq} V_{aq} + C_{org} V_{org} \quad (1)$$

where V_{aq} and V_{org} are the volumes in the water and organic phase, respectively, and C_{org} is the concentration of catechin in the organic phase at the partition equilibrium. Equation (1) is converted to Eqn. (2), and C_{org} is calculated from the measured C_{aq} .

$$C_{org} = (C_{aq, 0} - C_{aq}) V_{aq} / V_{org} \quad (2)$$

The partition coefficient, P , of catechin between methyl octanoate and water is defined as Eqn. (3).

$$P = C_{\text{org}} / C_{\text{aq}} \quad (3)$$

The measurement of the partition coefficient was done in duplicates, and the mean value was calculated.

3. Results and Discussion

3.1 Degradation of catechin at different concentrations

Figure 1 shows the transient changes in the fractions of remaining catechin, C/C_0 , at the initial concentration of 1.0, 2.5 and 10 mmol/L in an aqueous solution at 37°C and pH 6.5. C_0 and C represent the initial concentration of catechin and that at time, t , respectively. The fraction was greater than 0.5 after 6 days at each concentration. The degradation kinetics of the catechin was empirically expressed by the Weibull equation, which is flexible and can be used to describe many deterioration kinetics [16]:

$$C/C_0 = \exp[-(kt)^n] \quad (4)$$

where k is the rate constant, the reverse of which is called the scale parameter, and n is the shape constant. The kinetic parameters, k and n , were evaluated by fitting the experimental results by a nonlinear regression using Solver of Microsoft® Excel 2003. The curves in Fig. 1 were drawn based on the equation using the estimated

parameters. The R -squares for 1.0, 2.5 and 10 mmol/L of catechin were 0.990, 0.981 and 0.838, respectively. Figure 2 shows the relationships between the rate constant or the shape constant of the Weibull model for the degradation of catechin and its concentration at 37°C and pH 6.5. Both the k and n values decreased with the increase in the initial concentration, and especially the values were low at the concentration of 10 mmol/L. However, the goodness of fit of the Weibull model for the degradation of 10 mmol/L catechin was low. The n value for each concentration was less than 1, which indicated that the fraction of remaining catechin rapidly decreased during the early stage of storage and its decrease gradually became smaller. These results showed that the fraction of remaining catechin more slowly decreased at the higher initial concentration. The degradation process of catechin was thereafter measured at the concentration of 1.0 mmol/L.

3.2 Effects of pH and temperature on the degradation of catechin

Figure 3 shows the degradation processes of catechin at 37, 50, 60 and 70°C and pH 5.0, 6.0, 6.5, 7.0 and 7.5. The degradation of catechin proceeded fast at high temperature and pH in the aqueous solution. These trends were same as the previous reports [12,14]. Each degradation process was fully expressed by the Weibull model. Figure 4 shows the temperature dependencies of the k

Fig. 1 The transient changes in the fractions of remaining catechin at the concentration of (○) 1.0, (□) 2.5 and (△) 10 mmol/L in an aqueous solution at 37°C and pH 6.5. The solid curves were calculated using the estimated kinetic parameters of the Weibull model.

Fig. 2 Relationships between (a) the rate constant, k , or (b) the shape constant, n , of the Weibull model for the degradation of catechin and its concentration at 37°C and pH 6.5. The solid curves were empirically drawn.

Fig. 3 Degradation processes of catechin at (a) 37°C, (b) 50°C, (c) 60°C and (d) 70°C and (○) pH 5.0, (□) pH 6.0, (△) pH 6.5, (◇) pH 7.0 and (▽) pH 7.5. The solid curves were calculated using the estimated kinetic parameters of the Weibull model.

and n values for the degradation of catechin at various pHs. The k value was higher at the higher temperatures. The Weibull model has characteristics of a sigmoidal pattern that can be described when $n > 1$, that the model expresses the simple first-order kinetics at $n=1$, and

that C/C_0 steeply decreases during the early stage when $n < 1$. All the n values at tested pHs were smaller than unity. There was no remarkable tendency for the n value at every pH, but the n value seemed to decrease with increasing temperature.

The temperature dependence of the rate constant k was analyzed based on the Arrhenius equation:

$$k = k_0 \exp(-E / RT) \quad (5)$$

where k_0 is the frequency factor, E is the apparent activation energy, R is the gas constant, and T is the absolute temperature. The Arrhenius plots in Fig. 4(a) produced a straight line to evaluate the E and k_0 values from the slope and the intercept of the line, respectively. It would be expected that the enthalpy-entropy compensation held during the decomposition. Equation 6 is one of the expressions describing the compensation [17–19].

$$E = RT_\beta \ln k_0 + \gamma \quad (6)$$

where T_β is an isokinetic temperature and γ is a constant. The E values are plotted versus the logarithms of the k_0 values in Fig. 5. The plots lie on a straight line ($R^2 = 0.978$), suggesting that each degradation of catechin at the various pHs essentially proceeded by the same mechanism. The T_β value was estimated to be 46.4°C from the slope.

Fig. 4 Temperature dependencies of (a) the rate constants, k , and (b) the shape constants, n , of the Weibull model for the degradation of catechin. The symbols are the same as in Fig. 3. The solid curves were empirically drawn.

Fig. 5. Relationship between the apparent activation energy, E , and the logarithm of the frequency factor, k_0 , for the rate constant of the Weibull model. The k_0 value is in units of h^{-1} .

3.3 Comparison between ascorbic acid and octanoyl ascorbate for the catechin degradation in aqueous solution

Chen *et al.* reported that ascorbic acid stabilized green tea catechins [12]. Further, apparent first order reaction rate of total catechins, such as (–)-epicatechin, (–)-epicatechin gallate, (–)-epigallocatechin and (–)-epigallocatechin gallate, in green tea with ascorbic acid was compared with that without it [14]. (+)-Catechin solution, not green tea extracts, was selected as a simple system to investigate antioxidative property of acyl ascorbate against a water-soluble and unstable compound in aqueous solution. Figure 6 shows the transient changes at 60°C and pH 6.0 in the fractions of remaining catechin in the aqueous solution with ascorbic acid and octanoyl ascorbate at the concentrations of 0.001, 0.005, 0.01, 0.1, 1, 2 and 5 mmol/L. The Weibull model was applied to these results. The R^2 values for 0.001, 0.01, 0.1 and 1 mmol/L of ascorbic acid were 0.886, 0.947, 0.973 and 0.901, and those for 0.001, 0.005, 0.01, 0.1, 1, 2 and 5 mmol/L of octanoyl ascorbate were 0.986, 0.983, 0.938, 0.956, 0.945, 0.938 and 0.992, respectively. The stability of catechin at every tested concentration of ascorbic acid was higher than that without the acid. For the catechin solution with ascorbic acid, it was observed that the stability was higher at the lower concentration of the acid. On the other hand, the stability of catechin with octanoyl ascorbate at concentrations less than 0.01 mmol/L was higher than that without the ascorbate, and that at concentrations greater than 0.1 mmol/L was lower. The

Fig. 6 Degradation processes at 60°C and pH 6.0 of catechin with (a) ascorbic acid and (b) with octanoyl ascorbate at the concentration of (○) 0.001, (▽) 0.005, (□) 0.01, (△) 0.1, (◇) 1, (■) 2 and (▲) 5 mmol/L. The degradation processes of catechin with 0.005, 2 and 5 mmol/L ascorbic acid were not measured. The solid and broken curves were calculated using the estimated kinetic parameters of the Weibull model for the degradation processes of catechin with and without the ascorbates, respectively.

dependency of the estimated k value on the concentration of ascorbic acid or octanoyl ascorbate is shown in Fig. 7. The adequacy of the Weibull model for the degradation processes of catechin with 0.001 and 1 mmol/L of ascorbic acid was a little low, but the dependency in the case of octanoyl ascorbate was obviously different from that for ascorbic acid. That is, octanoyl ascorbate showed its characteristic behavior as an antioxidative surfactant in aqueous solution. The antioxidative property of octanoyl ascorbate against catechin in an aqueous solution may be due to formation of the micelle for octanoyl ascorbate. The critical micelle concentration of octanoyl ascorbate in an aqueous solution was 1.24 mmol/L at 25 °C [7]. The partition of catechin from water to methyl octanoate, which was assumed to be the micelle phase formed by the octanoyl moiety of the ascorbate, was measured at 37°C. The inset in Fig. 7 shows the relationship between the partition coefficient, P , and the initial concentration of catechin in the water phase. The P value was higher at the higher catechin concentrations, though it was not more than 0.1 at 100 mmol/L. As the P value was very low, the partition of catechin to the micelle would not result in the apparent catechin decrease. Although the reason why octanoyl ascorbate acted on the catechin degradation as an antioxidant or a prooxidant at the turning point of 0.1 mmol/L remains unclear, the for-

Fig. 7 Effect of the concentration of (●) ascorbic acid or (○) octanoyl ascorbate on the rate constant, k , at 60°C and pH 6.0. The broken line represents the k value for the degradation of catechin with no additive at the corresponding conditions. The inset shows the relationship between the partition coefficient (methyl octanoate/water) at 37°C and the initial concentration of catechin. The solid curves were empirically drawn.

mation of the micelle for octanoyl ascorbate may prevent it from suppressing the oxidative degradation of catechin in an aqueous solution.

3.4 Antioxidative property of octanoyl ascorbate against catechin in an aqueous solution at various temperatures

Figure 8 shows the degradation processes of catechin with 0.001 mmol/L of octanoyl ascorbate at 37, 50, 60 and 70°C and pH 6.0. The R^2 values on the Weibull model for 37, 50, 60 and 70°C were 0.976, 0.990, 0.986 and 0.851, respectively. The application of the Weibull model to the degradation at 70°C seemed to be difficult. The octanoyl ascorbate retarded the degradation of catechin at each temperature, and the antioxidative effect of the ascorbate was observed over a wide temperature range. Especially, the degradation was strongly suppressed at 37°C, and the fraction of remaining catechin was greater than 0.9 at 24 h. Figure 9 shows the relationship between the remaining fractions with and without the octanoyl ascorbate at 50, 60 and 70°C. Regardless of temperature, the plots were linear in Fig. 9 ($R^2=0.900$), indicating that the antioxidative action of octanoyl ascorbate on catechin in an aqueous solution was independent of temperature.

Conclusion

It was kinetically indicated using the Weibull model that the stability of catechin in aqueous solution depended on the concentration, pH and temperature. Hold of the enthalpy–entropy compensation during the degradation of catechin suggested that each degradation at tested pHs essentially proceeded by the same mecha-

Fig. 8 Degradation processes of catechin with 0.001 mmol/L of octanoyl ascorbate at (a) 37°C, (b) 50°C, (c) 60°C and (d) 70°C and pH 6.0. The solid and broken curves were calculated using the estimated kinetic parameters of the Weibull model for the degradation processes of catechin with and without octanoyl ascorbate, respectively.

Fig. 9 Relationship between the remaining fractions, C/C_0 , of catechin with and without octanoyl ascorbate at (□) 50°C, (△) 60°C and (◇) 70°C and pH 6.0.

nism. The dependency of the rate constant for the degradation of catechin in the presence of octanoyl ascorbate on the concentration was different from that for the degradation with ascorbic acid, and octanoyl ascorbate acted as both antioxidant and prooxidant depending on the concentration. The micelle formation of octanoyl ascorbate might prevent it from suppressing the oxidative degradation of catechin in an aqueous solution. Furthermore, it was shown that the antioxidative action of octanoyl ascorbate on catechin in aqueous solution was independent of temperature, but further research about the behavior of acyl ascorbate in aqueous solution is needed.

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◆◆◆ 和文要約 ◆◆◆

アスコルビン酸またはオクタノイルアスコルビン酸存在下における水溶液中でのカテキンの分解動力学

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ビタミンCとして知られるL-アスコルビン酸は、強い還元能をもつため広く利用されている水溶性抗酸化剤である。有機溶媒中でリパーゼの触媒作用を利用したアスコルビン酸と脂肪酸との縮合反応により、6-O-アシルアスコルビン酸が合成されている。アシルアスコルビン酸は親水性基と親油性基を有する両親媒性の抗酸化剤であり、抗腫瘍活性などを有するため有益な食品添加物であるといえよう。著者らも、固定化リパーゼを用いた回分および連続反応にて各種アシルアスコルビン酸を合成し、それらの界面活性剤特性や脂質に対する抗酸化能を評価するとともに、脂質粉末化技術への適用を検討した。しかしながら、アシルアスコルビン酸の効果的な利用にはその水溶液中での挙動についての知見が必要であるにも関わらず、水溶液中での抗酸化特性に関する検討はなされていない。一方、生理的に有用な物質であるカテキンを含むほとんどの茶飲料は、寒冷期間中はベンダー内で加熱保存され、そのうえ好熱性嫌気性菌の胞子を死滅させるために高温での熱処理が必要であるため、水溶液中での茶カテキンの安定性向上は重要な課題といえる。本研究では、水溶液中での(+)α-カテキンの分解過程を様々なpHおよび温度下で測定した。そして、その分解過程をWeibull式で表現し動力学パラメータを決定した。固定化リパーゼを用いてアセトン中でアスコルビン酸とオクタノ酸を縮合させ、アシルアスコルビン酸の中では比較的親水的なオクタノイルアスコルビン酸を合成した。そして、アスコルビン酸またはオクタノイルアスコルビン酸を添加した場合の水溶液中でのカテキンの分解動力学について検討した。

オクタノイルアスコルビン酸の合成には、*Candida antarctica* 由来の固定化リパーゼを用い、2-ブタノンおよび水による二相分配抽出にて精製した。所定量のカテキン、アシルアスコルビン酸およびリン酸緩衝液(pH5.0~7.5)を茶褐色バイアル瓶に入れ、所定温度(37~70℃)の恒温水槽に浸し振盪させた。ODSカラム(4.6

mmφ×250 mm)およびUV検出器(280 nm)を用いたHPLCにてカテキン残存量を測定した。

カテキン分解について動力学解析を行った結果、カテキン初濃度が高いほどWeibull式の適合性が低かったが、分解速度定数 k および形状定数 n がいずれも小さかった。いずれの初濃度でも n 値が1未満であったため、初期段階で分解が進み、徐々に分解速度が低くなることが示された。これらの結果は、カテキン初濃度が高いほど、カテキンの残存分率の低下が遅いことを示している。また、pHおよび温度が低いほど k 値が低く、低温・低pHでのカテキンの高い安定性が示された。一方、 n 値には顕著なpHおよび温度依存性が認められなかった。速度定数の温度依存性がArrhenius式に基づいて解析され、見かけの活性化エネルギーおよび頻度因子を決定した結果、カテキン分解過程におけるエンタルピー-エントロピー補償の成立が示されたため、異なるpH下での分解が本質的に同じメカニズムで進んだことが示唆された($T_\beta=46.4^\circ\text{C}$)。カテキン水溶液へのアスコルビン酸添加によりその安定性が向上し、アスコルビン酸濃度が低いほど k 値が低くなる傾向が認められた。他方、オクタノイルアスコルビン酸を添加した場合では、その濃度が0.01 mmol/L以下であればカテキン分解が抑制され、0.1 mmol/L以上であれば分解速度が高くなった。オクタノイルアスコルビン酸の25℃水溶液中での臨界ミセル濃度が1.24 mmol/Lであるため、オクタノイルアスコルビン酸のこの特徴的な挙動がミセル形成に起因するのではないかと考え、水-オクタノ酸メチル系でのカテキンの分配係数 P を測定した。カテキン濃度が高いほど P 値は高かったが、いずれのカテキン濃度でも0.1未満であった。したがって、カテキンのミセル相への分配による見かけのカテキン量の減少ではないことが確認された。しかし、オクタノイルアスコルビン酸のミセル形成がカテキンの不安定化に何らかの影響を与えているものと考えられる。異なる温度下におけるオクタノイルアスコルビン酸添加時のカテキンの安定性を測定した結果、オクタノイルアスコルビン酸はいずれの温度でもカテキン分解を抑制し、その抗酸化能は温度に依存しないことが示された。

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