1,5-アンヒドロフルクトースの調製と抗酸化活性

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Preparation and Antioxidative Activity of 1,5-Anhydrofructose

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A simple enzymic method for the preparation of 1,5-anhydrofructose (1,5-AF) and its antioxidative activity were investigated. α-1,4-glucan lyase (EC 4.2.2.13, GLase) was extracted and purified from red seaweed, *Gracilaria verrucosa*. One-hundred-and-fifty grams of 1,5-AF was obtained from 360 g of waxy maize starch by digestion with the purified GLase and the following purification of the product by ion-exchange resins and gel filtration. The purity of the product was 98.6% by HPLC using an ion-exchange resin (MCI GEL CK08S). The product, 1,5-AF, was identified by ^{1}H-^{13}C COSY NMR, and the $[\alpha]_D^{25}$ was $-16.8°$. The antioxidative activity of 1,5-AF was examined by comparison with that of ascorbic acid (VC) for preventing the oxidation of linoleic acid. The antioxidative activity of 1,5-AF was found to be approximately 1.8-fold that of VC by the methods using thiocyanate and 2-thiobarbituric acid.

1,5-Anhydrofructose (1,5-anhydro-D-arabino-hex-2-ulose, 1,5-AF) was first isolated from *Morchella vulgaris* as the precursor of the pyrone microthesin,[1] and then in red alga, *Gracilariopsis lemaneiformis*.[2,3] 1,5-AF is produced from starch and glycogen by degradation with an enzyme α-1,4-glucan lyase (EC 4.2.2.13, GLase).[2-5] 1,5-AF has been found in various living organisms, such as fungi,[4] and algae,[2,6] as well as in rat livers and other organs.[7] 1,5-AF has been suggested to be the initiator of the third glycolytic pathway as the precursor of 1,5-anhydroglucitol,[5,7] and it may be connected somehow to control the blood glucose level.

The carbonyl group of 1,5-AF is not involved in hemiacetal bonding as in other reducing sugars. 1,5-AF has been suggested to be in an equilibrium mixture in an aqueous solution comprised of 2,3-endiol, 2-enol and 2-keto forms, and the hydrated form (Fig. 1), and it shows considerably stronger reducing power than the general pentoses and hexoses.[6] The 2,3-endiol form may be the most reactive.[6] This high reducing power suggests the possible use of 1,5-AF as an antioxidant. In this study, we investigated a simple method for the preparation of 1,5-AF and its antioxidative activity.

Antioxidants are known to play an important role *in vivo* to scavenge active oxygen, which may induce cancer and high blood pressure through the oxidation of cholesterol. Antioxidants are very useful in the food industry to prevent oxidation of foodstuffs, lipids, flavors, pigments, *etc*. Several natural antioxidants, extracted from tea, vegetables, fruits, *etc*., are widely used but are rather expensive.

MATERIALS AND METHODS

Materials. All of the chemicals were of the highest grade available commercially. Waxy maize starch was purchased from Honen Corporation (Tokyo, Japan) and dextrin (DE 10) was a product (Loremalt 2002E) of Lorenz Co. (Blumenau, Brazil). Ion-exchange resigns, IR-120 and IRA-68, were obtained from Organo Co. (Tokyo, Japan). TOYO PEARL HW-40S was purchased from Tosoh Co. (Tokyo, Japan). A

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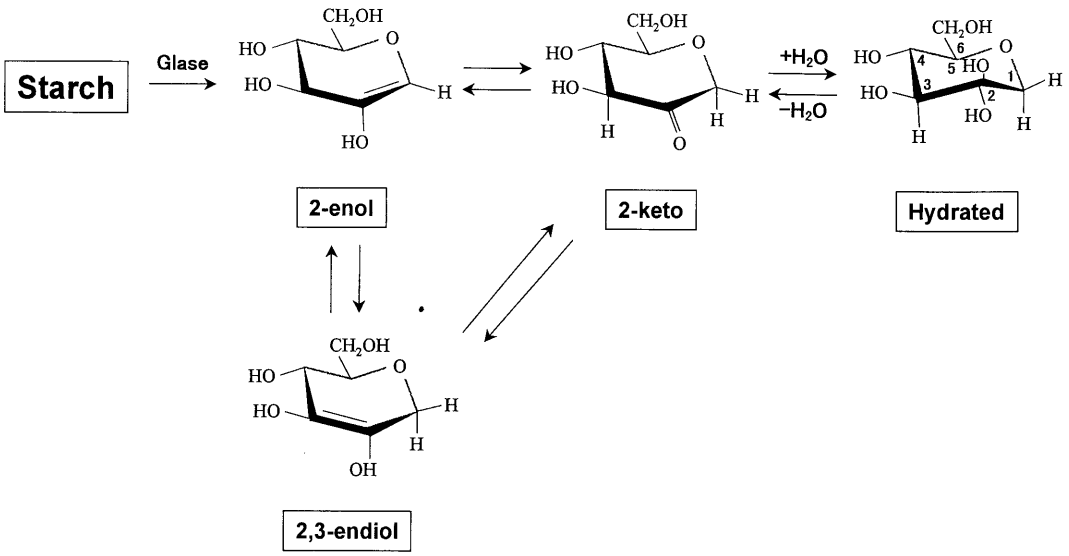

Fig. 1. Production of 1,5-AF from starch and the possible structures of 1,5-AF in water.

fresh red seaweed, *Gracilaria verrucosa,* which was harvested in Ariake Bay, Japan, during June 1997, was purchased from Kaneryou Co. (Kumamoto, Japan) and stored at −35℃ until use.

NMR spectroscopy. 1,5-AF was dissolved in D_2O (10%) and NMR spectra were recorded at 50℃ with a JEOL GSX-500 spectrometer. The chemical shifts, expressed in parts permillion (ppm), downfield from the signal of tetramethylsilane (Me_4Si) referred to external 1,4-dioxane (67.40 ppm).

Specific optical rotation measurement. Specific optical rotation, $[\alpha]_D$, was measured in an aqueous solution (1 g/100 mL H_2O) at 25℃ by a polarimeter [JASCO P1030].

Determination of protein. Protein was assayed by the method of Bradford.[8]

SDS-gel electrophoresis. SDS-gel electrophoresis of the purified enzyme (1.0 μg) was carried out by the method of Laemmli.[9]

Assay of GLase. GLase activity was assayed by a method similar to that reported by Yu *et al.*[2] Appropriately diluted enzyme solution (50 μL) was added to 0.9% soluble starch in 50 mM acetate buffer, pH 5.5, and the mixture was incubated at 35℃ for 30 min. The reaction was terminated by adding 1 mL of the alkaline 3,5-dinitrosalicylic acid reagent (0.89% sodium hydroxide containing 0.56% 3,5-dinitrosalicylic acid and 1.67% sodium potassium tartrate), and incubated at 35℃ for 15 min. The color developed was measured by a spectrophotometer at 546 nm, and the amount of 1,5-AF was determined by using purified 1,5-AF, purity 98.6%, as a standard.

Assay of antioxidative activity by the 2-thiobarbituric acid (TBA) method.[10] Either 1 or 10 mg of 1,5-AF or 10 mg of ascorbic acid (VC), as a reference, was added to 10 mL of an aqueous solution containing 4.5 mM linoleic acid and 0.4% Tween 40. The control run was carried out on a solution containing linoleic acid but not 1,5-AF or VC. The reaction mixtures were sealed in test tubes and incubated at 50℃ for 16 days. Two milliliters of the solution was taken out at appropriate intervals, 2 mL of 20% trichloroacetic acid and 1 mL of 0.67% TBA were added, and then the solution was heated at 100℃ for 10 min. After cooling the solution to room temperature, the resulting precipitate was centrifuged off. Then, the formation of TBA-chromogen, which linked TBA and malonaldehyde produced by the oxidation of linoleic acid, was measured at 532 nm (A_{532}). The antioxidant activity was calculated as the amount (%) of unoxidized linoleic acid remaining, and that of the control run was 0%.

Assay of antioxidative activity by the ferric thiocyanate method.[11] Two milliliters of 99.5% ethanol containing 2.8% (w/v) linoleic acid (w/v), 4 mL of 50 mM sodium acetate buffer (pH 7.0), 2 mL of 75% (v/v) ethanol containing antioxidants, and 2 mL of distilled water were mixed in test tubes that were then sealed with parafilm. The control run was the mixture containing no antioxidants. After incubation at 40℃ for 7 days in darkness, 0.1 mL of each mixture was taken out and 9.7 mL of 75% ethanol and 0.1 mL of 30% ammonium thiocyanate solution were added. After 3 min, 0.1 mL of hydrochloric acid (3.5%) containing 20 mM ferrous chloride was added to the mixture. The absorbance of the solution at 500 nm (A_{500}) was measured and the antioxidative activity was expressed as the amount (%) of unoxidized linoleic acid remaining, and that of the control run was 0%.

HPLC analysis. The purity of 1,5-AF was analyzed by HPLC (Shodex DS-4) using a MCI GEL CK08S column (8.0×500 mm, Mitsubishi Chemical Co.) and a differential refractometer (Shodex RISE-61). The flow rate of eluent water was 1.0 mL/min.

RESULTS AND DISCUSSION

Preparation of GLase.

Red seaweed, *Gracilaria verrucosa*, was one of the choice sources of GLase because it contains relatively abundant enzyme (6 U/g fresh alga) and is commercially available. *Gracilaria chorda* was also a good source of enzyme (4–5 U/g fresh alga) and the seaweed is easily collected along the coast of southern Kagoshima, Japan. The enzyme activity of the seaweed was unvaried during the harvest season from January to June. One-hundred grams of *Gracilaria verrucosa* was homogenized well with 200 mL of 20 mM sodium acetate buffer, pH 5.5 (buffer A),[1] at 4℃ by a homogenizer (ULTRA TURRAX T50, IKA). The homogenate was centrifuged at 3000×*g* for 30 min and the supernatant (crude extract) was collected. The residue was extracted twice as above with buffer A. The total enzyme activity in the combined crude extracts (*ca*, 2 L) was 3140 U. The enzyme was purified by its high affinity to starch.[2] The suspension (2 mg/mL) of waxy maize starch in 800 mL of 50 mM sodium acetate buffer, pH 5.5 (buffer B), was added to the enzyme extract while stirring. The mixture was stirred gently overnight to complete absorption of the enzyme by the starch. Then the starch was washed thoroughly with buffer B by suspending and centrifuging repeatedly until no more protein could be detected. The enzyme was eluted from the starch by washing the starch with 200 mL of buffer A containing 20 mg/mL dextrin (DE 10) and stirring gently overnight. After the starch was removed by centrifugation, the supernatant was dialyzed against 1 liter of buffer B with occasional replacement by fresh buffer for 12 h at 4℃. Turbid materials produced were removed by centrifugation, and the supernatant, which contained the 1870 U enzyme, was used for the preparation of 1,5-AF. The yield of the enzyme was 60% and its specific activity was 99.7 U/mg protein, which increased 59.7-fold from the crude extract. The specific activity is a similar value to that of the purified enzyme by Yu *et al.*[2] The enzyme preparation gave a single band by SDS-gel electrophoresis.[9]

Preparation of 1,5-AF.

Waxy maize starch (360 g) was suspended in water (11 L) and heated at 100℃ until it made a clear paste. The pH of the solution was adjusted to 5.5 with 1 M acetic acid. Buffer B (200 mL) containing GLase (3600 U) was added to the waxy maize paste, and the mixture was incubated at 35℃ for 48 h. Sixty-two percent of the starch was converted to 1,5-AF during the incubation period (Fig. 2). The production appeared to be at the limit because 1,5-AF did not increase with extended incubation. The enzyme is unable to bypass the branch linkages in starch because that the glucosyl residues at the non-reducing ends of 6^2-α-glucosylmaltotriose are not liberated.[12] An equal volume of ethanol was added to the mixture and the mixture was left standing at room temperature for 1 h to precipitate the remaining substrate. After centrifugation of the precipitate, the supernatant was concentrated to 30% (w/w)

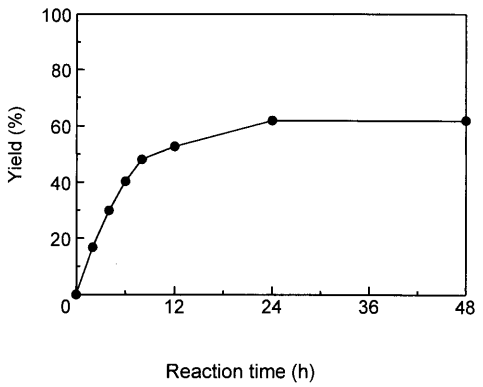

Fig. 2. Time course for the production of 1,5-AF from waxy maize starch.

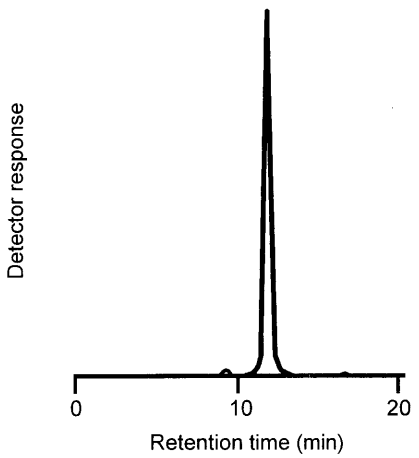

Fig. 3. HPLC chromatogram of 1,5-AF.

and then the solution was applied successively to columns (1×10 cm) packed with ion-exchange resins, IR-120 and IRA-68 (Organo), to remove salts. The eluate was applied to an HW-40S (Toyopearl) gel-filtration column (5× 100 cm) to remove the remaining oligo- and polysaccharides. The eluate fractions containing 1,5-AF were combined, lyophilized, and stored in a refrigerator until use. One-hundred-fifty grams of 1,5-AF was obtained. The yield was 42%. The above procedures are as simple as those used for the production of glucose or maltose from starch because the preparation requires only one enzymic process. The purity of the lyophilized product was 98.6% by HPLC as shown in Fig. 3.

The yield of 1,5-AF increased when isoamylase was added together with GLase. However, the cooperative action of isoamylase and GLase produced glucose from the reducing end of the debranched amylopectin chains. The resulting glucose decreased the purity of the final product, therefore the glucose had to be removed by means of bakers yeast.[4] In preliminary tests, we used a crude extract of the enzyme, but it was not suitable because several unknown by-products or impurities accompanied by the enzyme were detected by HPLC. The purified enzyme gave 1,5-AF almost exclusively as shown in Fig. 3, and it was possible to prepare reasonably pure 1,5-AF without any tedious process.

Identification of 1,5-AF.

The final product was identified as 1,5-AF by ^{1}H-^{13}C COSY NMR spectroscopy. All of the carbon atoms were assigned by the splitting of proton signals linked to the carbon as shown in Fig. 4. Two methylene carbons, C-1 and C-6, appeared in opposite phases of C-3 to C-5 and the C-2 linking two OH was undetectable by the DEPT method (spectrum not shown). The chemical shifts (ppm) of C-1, C-2, C-3, C-4, C-5, and C-6 were 74.45, 95.33, 79.70, 71.78, 83.32, and 63.96, respectively. A signal at 208.56 ppm, which appeared to belong to the keto form, was found also but it was minor and less than 10% of the material judging from the intensity, and the signals at around 130-150 ppm due to the double bond(s) were not detectable (data not shown). These data suggest that the product was 1,5-AF and the major form of 1,5-AF in water is the hydrated form (Fig. 1) as reported by previous workers.[2,12-15] Specific optical rotation, $[\alpha]_D^{25}$, was found to be $-16.8°$.

Antioxidative activity of 1,5-AF.

The antioxidative activity of 1,5-AF measured by the TBA method[10] is shown in Fig. 5. 1,5-AF at the 0.01% level prevented the oxidation of linoleic acid more effectively than the same concentration of VC throughout the incubation period. The antioxidative activity of 1,5-AF after 16 days was estimated to be about 1.8-fold as strong as that of VC. The antioxidative

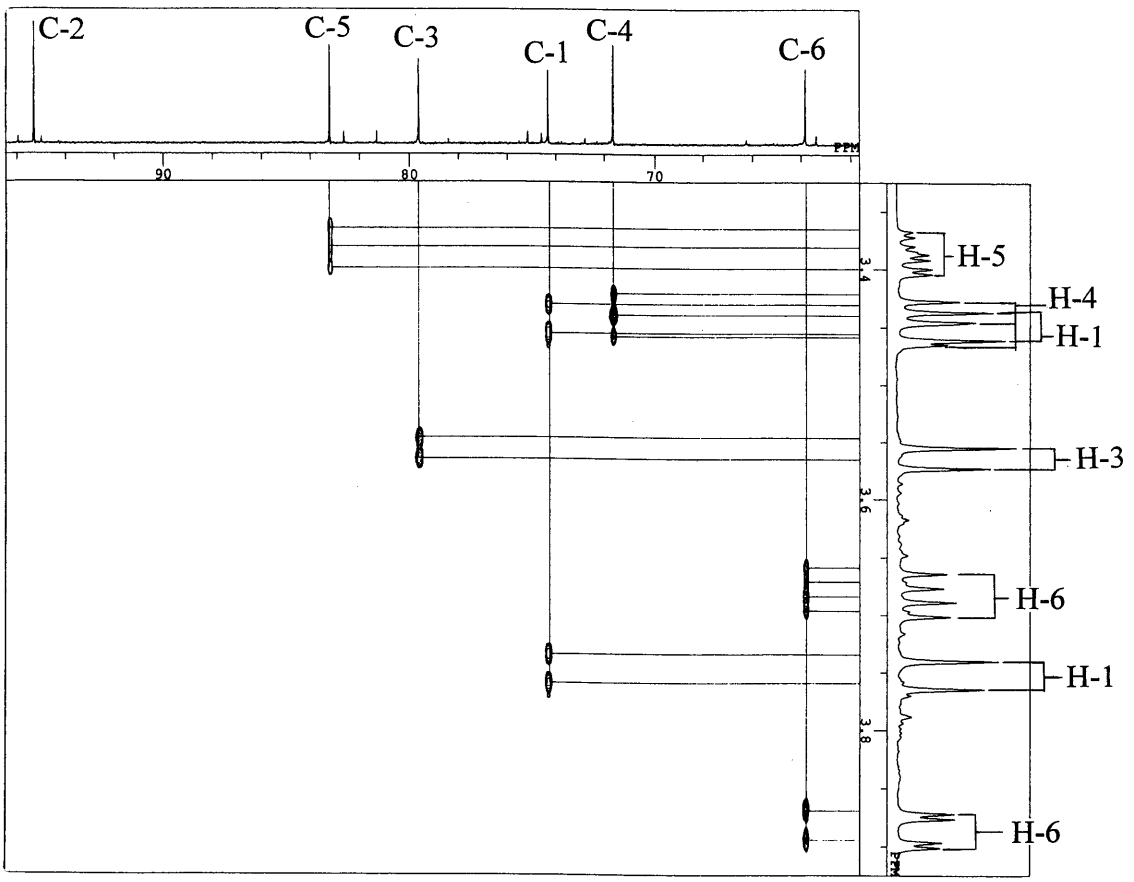

Fig. 4. ^{1}H-^{13}C COSY NMR spectrum of 1,5-AF in D_2O at 500 MHz.

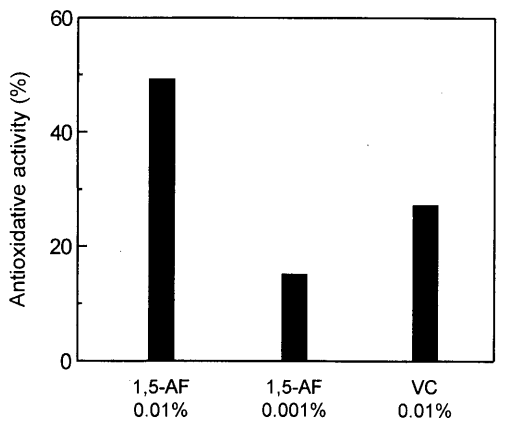

Fig. 5. Antioxidative activities of 1,5-AF and VC measured by the TBA method.[10]

For details, see text.

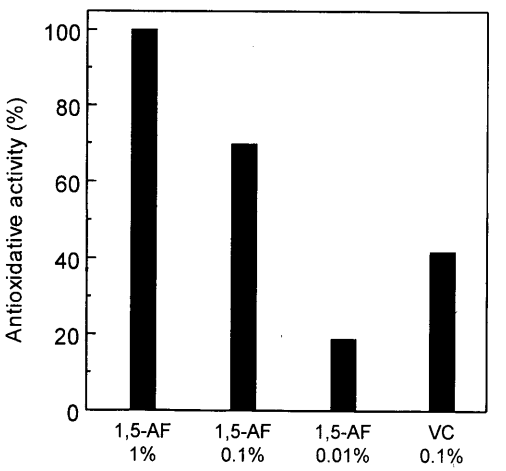

Fig. 6. Antioxidative activities of 1,5-AF and VC measured by the ferric thiocyanate method.[11]

For details, see text.

activity of 1,5-AF was also examined by the ferric thiocyanate method.[11] Figure 6 shows that 0.1% of 1,5-AF prevented the oxidation of linoleic acid 1.7-fold stronger than the same concentration of VC after incubation for 7 days, similar to the case measured by the TBA method.

Uses of 1,5-AF.

These results suggest that 1,5-AF has an antioxidative activity higher than that of VC and it appears to be a useful antioxidant. In addition, it is coloress, odorless, and neutral, and seems to be capable of being produced economically. Therefore, it is expected that 1,5-AF can be used widely as an antioxidant in the fields of food, medicine and other industries.

CONCLUSION

1,5-AF was found to be a stronger antioxidant than VC and could be produced from starch by simple procedures of degradation with the GLase of a red seaweed *Gracilaria verrucosa*. 1,5-AF can be used widely in food and other industries.

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1,5-アンヒドロフルクトースの調製と抗酸化活性

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1,5-アンヒドロフルクトース (1,5-AF) の簡便な調製法を開発し，その抗酸化活性を調べた．まず，α-1,4-グルカンリアーゼ (EC 4.2.2.13) を紅藻類の *Gracilaria verrucosa* (おごのり) から抽出し，澱粉吸着により精製した．この酵素を用いて，360 g のモチトウモロコシ澱粉からイオン交換樹脂とゲル濾過の行程を経て，150 g の純度 98.6%の 1,5-AF を得た．産物は ^{1}H, ^{13}C COSY NMR によって 1,5-AF と同定した．$[\alpha]_D^{25}$ は −16.8° であった．1,5-AF の抗酸化力をリノール酸の酸化防止を対象として，アスコルビン酸 (VC) と比較検討した．その結果，1,5-AF は VC に対して，チオバルビツール酸法やチオアシン酸法では，ほぼ 1.8 倍の抗酸化力のあることが認められた．