The Hydrolysis Mechanism of Inulin and Its Hydrolysate in the Reaction Field by the Hot Compressed Water

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Aiming to produce functional oligosaccharides such as di-D-fructose dianhydrides (DFAs) from inulin in subcritical water (the hot compressed water), the hydrolysis mechanism of inulin was investigated. The hydrolysis of inulin was conducted in carbon dioxide-the hot compressed water (CO₂-HCW) created with a batch-type reactor (temperature: 100–150°C, pressure: 3.0–5.0 MPa). The inulin hydrolysate prepared with CO₂-HCW were determined by electrospray ionization mass spectrometry and UV-Vis spectroscopy. The results showed that inulin is hydrolyzed to monosaccharides, disaccharides, fructooligosaccharides, and DFAs with CO₂-HCW. The hydronium ion concentration ([H⁺]) formed in CO₂-HCW was approximately estimated 100-fold higher than [H⁺] created in the hot compressed water without CO₂. High [H⁺] is believed to be one of the factors that contributes to the hydrolysis of inulin <150°C. The generation of 5-hydroxymethylfurfural (HMF) occurred by intramolecular dehydration of fructose was suppressed due to low temperature process (<150°C). CO₂-HCW is one of the means to convert inulin into DFAs while suppressing the generation of HMF.

Keywords : carbon dioxide, di-D-fructose anhydrides, electrospray ionization mass spectrometry, hydronium ion, inulin, the hot compressed water

INTRODUCTION

Chicory (*Cichorium intybus* L.) is investigated to produce in Tokachi district, Hokkaido, Japan or some plant factories. The roots of chicory contain inulin; it is polysaccharide composed of β -(2,1)-linked β -D-fructofuranosyl residues and terminated with α -D-glucopyranosyl unit through α -(1,2) glycosidic linkage. In plants, it is principally biosynthesized from sucrose and is one of the principal stored forms of energy. Inulin is the raw material of functional oligosaccharides such as di-D-fructose dianhydrides (DFAs) that can be used as low-caloric sweeteners with low cariogenicity and prebiotic properties (Minamida et al., 2004; 2005a; 2005b; Mitamura and Hara, 2005; Minamida et al., 2006; Yoshimoto et al., 2013) and may also increase the absorption of essential minerals (Shigematsu et al., 2004; Hara et al., 2010; Nakamori et al., 2010), flavonoids (Matsumoto et al., 2007). The techniques for converting inulin into DFAs are almost performed with enzymatic reactions (Uchiyama et al., 1973; Haraguchi et al., 1988; Kawamura et al., 1988; Yokota et al., 1991; Cho et al., 1996; Park and Choi, 1996; Kang et al., 1998; Haraguchi et al., 2002; 2005; 2006; Zhao et al., 2011; Wang et al., 2015). The DFA producing microorganisms were summarized, 30 microbial strains have been isolated to produce DFAs via enzymatic reaction, but only four isomers have been obtained by means of this, including DFA III, DFA I, DFA IV and DFA V (Wang et al., 2015). While the

reactions are highly selectivity and proceed in mild temperature, culturing bacteria and purification of enzymes are the essential process and might be the bottleneck for manufacturing DFAs in enzymatic reactions.

The application of subcritical fluids reaction fields in food processing have attracted much attention due to their unique ability. The hot compressed water, heated to 100–374°C and pressurized to state liquid phase (Savage, 1999), is one of subcritical fluids reaction fields and is most commonly used medium because water is abundant and non-toxicity. It has characteristic to hydrolysis the substances without any catalyst due to increase of ion product of water ($K_w=[H^+][OH^-]$). In some cases, the inert gas such as nitrogen gas or carbon dioxide gas are used for pressurizing water in a batch-type reactor. Here, we call the reaction field pressurized with nitrogen gas N₂-the hot compressed water (N₂-HCW) or pressurized with carbon dioxide gas CO₂-the hot compressed water (CO₂-HCW). Nitrogen gas is not related to the reaction in N₂-HCW, so the reaction occurred in N₂-HCW is the only effect of water. On the other hands, CO₂-HCW is known to accelerate the acid-catalyzed reaction by the high-pressure CO₂/H₂O technology (Morais et al., 2014). CO₂ dissolved in water creates carbonic acid whose value of acid dissociation constant (pK_a) is lower than the that of pK_a of water and generates the hydronium ions easily (Hunter and Savage, 2003; 2008). The acid-catalyzed reaction accelerated by CO₂-HCW would bring about two benefits. Firstly, the reactions conducted with CO₂-HCW are expected to proceed in rela-

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tively low temperature compared with N₂-HCW. This would lead to control the reaction rigidly in terms of reducing by-products which produced at high temperature range. Secondly, CO₂-HCW does not require the acids such as sulfuric acid or hydrochloric acid that are needed to be neutralized with base after the reaction. In addition, CO₂ dissolved in water can be recycled as depressurizing the reaction fields.

Previously, we prepared the inulin hydrolyzate solutions containing DFAs with N₂-HCW at 160–170°C, however, those solutions also contain 5-hydroxymethylfurfural (HMF) (Itoh and Shimizu, 2014). HMF is generated from hexose with the thermal decomposition or high-temperature treatment such as pyrolysis or hydrothermal processing. Although HMF is considered to be valuable intermediate for fine chemicals and liquid fuels (Mukherjee et al., 2014), its separation and purification are complicated. Considering the purification and drying process after the preparation of DFAs, it is desirable to suppress the generation of HMF during preparation. Almost all the reaction of hexose to HMF depends on the preparation temperature of intramolecular dehydration, which means the lower temperature preparation might be the key factor to suppress the generation of HMF. The goal of the current study is to convert inulin into DFAs using CO₂-HCW preparation at lower temperature (< 150°C) so as to suppress the generation of HMF and reveal the hydrolysis mechanism of inulin with CO₂-HCW by electrospray ionization mass spectrometry (ESI-MS), UV-Vis spectroscopy and estimation for the hydronium ion concentration ([H⁺]).

MATERIALS AND METHODS

Preparation of the inulin hydrolyzate solutions with the hot compressed water fluids

The experimental procedure was similar to a method published previously (Itoh and Shimizu, 2014; Yoshioka and Shimizu, 2014) and described below. We used Orafit HP, a chicory inulin containing long fructosyl chains with an average degree of polymerization (DP) of over 23 (DKSH Japan KK, Tokyo, Japan). The hydrolysis of inulin was performed in a batch-type reactor equipped with a pressure-resistant vessel made from SUS-316 stainless steel and an inner container made from Teflon and equipped with a type-K thermocouple and a pressure gauge. Inulin (0.1 g), distilled water (10 mL) and a magnetic stir bar were putted into the inner container and the vessel was pressurized to the appropriate pressure with nitrogen gas (N₂-HCW preparation) or carbon dioxide gas (CO₂-HCW preparation). The vessel was tightly closed and then heated and stirred at 1,000 rpm in an organic synthesizer (Chemi Station TM PPV-3000, Tokyo Rikakikai Co., Ltd., Tokyo, Japan) to the appropriate temperature, then held at the required temperature for 15 minutes. The temperature inside the vessel was monitored at 1 second intervals by using the type-k thermocouple. The temperature of the heating block and the pressure inside the vessel were monitored every 5 minutes. The temperature and the pressure changes in the HCW processes (CO₂-HCW and

Fig. 1 The change of temperature and pressure during the preparations. The temperature of the water (---) CO₂-HCW or (—) N₂-HCW, the temperature of heat block (●) or (■) and the pressure in the vessel (○) or (□). Circles and squares represent the preparation of CO₂-HCW and N₂-HCW, respectively.

N₂-HCW) was shown in Fig. 1. When the preparation was complete, the reaction was quenched by rapidly cooling the vessel with cooled water (5°C). The resulting inulin hydrolyzate solutions were collected in the glass vials and were kept at 5°C until they were analyzed.

Electrospray ionization mass spectrometry (ESI-MS)

The inulin hydrolyzate solutions were analyzed by using a mass spectrometer (Exactive; Thermo Fisher Scientific K.K., Kanagawa, Japan) equipped with an electrospray ionization (ESI) probe. The inulin hydrolyzate solutions were diluted with methanol. The ESI-MS experiments were performed in the negative-ion mode with scanning over the range of mass-to-charge ratios (*m/z*) 150–2,000. The following instrument settings were used: spray voltage 2.6 kV, sheath gas pressure 30 arbitrary units (a.u.), auxiliary nitrogen pressure 15 a.u., capillary temperature 300°C; heater temperature 250°C, capillary voltage –27.5 V, tube lens voltage –110 V, skimmer voltage –45 V.

UV-Vis spectroscopy

UV-Vis spectra of the inulin hydrolyzate were recorded on a UV-Vis spectrometer (V-560, Jasco Co., Ltd., Tokyo, Japan) in the wavelength range of 200 to 900 nm. The inulin hydrolyzate solutions (0.1 mL) were diluted with distilled water (4.9 mL) then the diluted solutions (4.0 mL) were poured into the standard cell made from synthetic quartz glass (S10, GL Sciences Inc., Tokyo, Japan).

Estimation of the hydronium ion concentration in the hot compressed water

Assuming hydronium ion (H⁺) and hydroxide ion (OH[–]) were ionized equally in the hot compressed water, [H⁺] was represented from equation (1) using the ion product of water ($K_w = [H^+][OH^-]$),

$$[H^+] = K_w^{1/2} \quad (1)$$

where the bracket represents the molal concentration. K_w was calculated from Marshall and Franck equation (Marshall and Franck, 1981), as shown below,

$$\log K_w = A + B/T + C/T^2 + D/T^3 + (E + F/T + G/T^2) \cdot \log \rho_w \quad (2)$$

where T is the absolute temperature of water (K), ρ_w is the density of water (g/cm^3), and the constants are: $A = -4.098$ (-), $B = -3,245.2$ (K), $C = 2.2362 \times 10^5$ (K^2), $D = -3.984 \times 10^7$ (K^3), $E = 13.957$ (-), $F = -1,262.3$ (K), $G = 8.5641 \times 10^5$ (K^2). The density of water was obtained from the NIST on-line chemistry web book (NIST, 2015). $[\text{H}^+]$ in N_2 -HCW were estimated from equation (1) and (2).

$[\text{H}^+]$ in CO_2 -HCW was estimated by the square root of the quantity of K_w plus a member of the contribution of CO_2 (Hunter and Savage, 2003;2008), as shown below,

$$[\text{H}^+] = (K_w + K_{a1} K_H P_{\text{CO}_2})^{1/2} \quad (3)$$

where K_{a1} , K_H , and P_{CO_2} represent the first ionization constant of carbonic acid (mol/kg), the Henry's law constant of carbon dioxide dissolved in water ($\text{mol}/(\text{kg MPa})$) and the pressure of carbon dioxide (MPa), respectively. K_{a1} was obtained from model 1 of Patterson et al. (Patterson et al., 1982), K_H was taken from Hunter and Savage (Hunter and Savage, 2008), and P_{CO_2} was obtained from the pressure gauge at reaction end.

RESULTS AND DISCUSSION

Electrospray ionization mass spectrometry

ESI-MS spectra (negative-ion mode) of the inulin hydrolyzate prepared by using CO_2 -HCW are shown in Fig. 2. The inulin hydrolyzate prepared at 100°C showed ions at $m/z = 179, 341, 503, 665, 827, 989, 1,151, 1,313, 1,475, 1,637, 1,799$ and $1,961$ (Fig. 2(a) and 2(b)). Mono-saccharides such as fructose (fr) or glucose (g) were observed at $m/z = 179$ ($[\text{fr}-\text{H}]^-$ or $[\text{g}-\text{H}]^-$). Disaccharides

such as fructodisaccharides (f_2) or sucrose (gf) formed the base peak at $m/z 341 = ([\text{f}_2-\text{H}]^- \text{ or } [\text{gf}-\text{H}]^-)$. Fructooligosaccharides (FOSs) with DPs of 3–12 produced deprotonated ions $[\text{f}_n-\text{H}]^-$ or $[\text{gf}_m-\text{H}]^-$ ($3 \leq n \leq 12, 2 \leq m \leq 11$) at $m/z = 503, 665, 827, 989, 1,151, 1,313, 1,475, 1,637, 1,799$ and $1,961$, respectively. At 110°C , FOSs with DPs of above 10 ($=m/z 1,799, 1,961$) were not observe. At 130°C , disaccharides formed the base peak at $m/z 341$ as well as shown in 100°C , however, FOSs with DPs above 5 ($=m/z 827$) were not observed. DFAs having a molecular mass of 324 Da also produced ions at $m/z 323$ $[\text{M}-\text{H}]^-$ or 647 $[2\text{M}-\text{H}]^-$ (M: DFAs) at 130°C . The cluster ion $[2\text{M}-\text{H}]^-$ is observed in ESI-MS when the substances are comparatively high concentration. At 150°C , DFAs formed the base peak at $m/z 323$ $[2\text{M}-\text{H}]^-$ and also produced the cluster ion at $m/z 647$ $[2\text{M}-\text{H}]^-$.

The preparations of CO_2 -HCW at 100°C had not influence on the hydrolysis of inulin as a result of the ESI-MS spectra of the inulin hydrolyzate, those including FOSs with DPs of 3–12. In contrast to 100°C , the hydrolysis of inulin proceeded to some extent at 130°C due to little FOS with DP_s of above 5. Therefore, the process temperature was found to be an important parameter to conduct the hydrolysis of inulin and effect on the inulin hydrolyzate components. ESI-MS spectrum of the inulin hydrolyze prepared at 150°C was very similar to that of DFA III standard as previously reported (Itoh and Shimizu, 2014). This result supports that DFAs are the major products of the hydrolyzate prepared at 150°C with CO_2 -HCW. The intramolecular dehydration of fructodisaccharides into DFAs occurs at temperatures above 160°C with N_2 -HCW. In the current study, however, the production of DFAs proceeds

Fig. 2 ESI-MS spectra (negative-ion mode) of the inulin hydrolyzate prepared at (a) 100°C , (b) 110°C , (c) 130°C and (d) 150°C .

Fig. 3 UV-Vis spectra of the inulin hydrolyzate prepared by (a) CO₂-HCW or (b) N₂-HCW.

at 150°C or less with CO₂-HCW. This is an important result the inert gas changing nitrogen gas to carbon dioxide gas allows to prepare DFAs at 130–150°C in subcritical fluid reaction fields.

The difference of [H⁺] in each reaction fields have probably influence on the extent of reaction. [H⁺] in CO₂-HCW would be expected to be much higher than [H⁺] in N₂-HCW. Considering the collision frequency between [H⁺] and inulin in each reaction fields, [H⁺] created in CO₂-HCW can easily attack to the β-(2,1) glycosidic bonds of inulin. Consequently, the hydrolysis of inulin in CO₂-HCW proceed at the temperature below 150°C.

UV-Vis spectroscopy

The UV-Vis spectra of the inulin hydrolyzate were shown in Fig. 3. The spectra prepared by CO₂-HCW at 100, 110 and 130°C were broad and no peaks were observed (Fig. 3(a)). At 150°C, the inulin hydrolyzate prepared by N₂-HCW provided the strong peak at 284 nm which might be corresponds to HMF or furfural, especially above at 160°C (Fig. 3(b)). Furthermore, the absorbance at 284 nm increased with temperature.

The absorbance at 284 nm of the inulin hydrolyzate prepared by N₂-HCW at 160, 165 and 170°C were higher than that of the ones prepared at 150 and 155°C. The absorbance depends on the concentration of HMF in the inulin hydrolyzate according to Lambert-Beer law. Thus, the concentration of HMF in the inulin hydrolyzate increases with preparation temperature. In CO₂-HCW, the intramolecular dehydration of fructose into HMF did not appear to proceed at 100, 110, and 130°C our experimental condition because of comparatively low temperature preparation, except for the conducted temperature at 130°C.

The temperature for the intramolecular dehydration of fructose into HMF seems to be different between CO₂-HCW and N₂-HCW at 150°C. This is also believed to be difference of [H⁺] in each reaction fields. Because [H⁺] in CO₂-HCW is much higher than N₂-HCW, the fructose as a result of hydrolysis in CO₂-HCW at 150°C is easily attacked from hydronium ions compared to the one occurred in N₂-HCW at 150 and 155°C. As a consequence, the intramolecular dehydration into HMF appears to slightly proceed in CO₂-HCW at 150°C. However, the absorbance of inulin hydrolysate prepared by CO₂-HCW at 150°C are lower than that of ones prepared by N₂-HCW at 160, 165 and 170°C. Therefore, CO₂-HCW can prepare DFAs from inulin with reduction of HMF.

Estimation of the hydronium ion concentration in the hot compressed water

The results of ESI-MS and UV-V is suggested that the ability of hydrolysis for inulin in CO₂-HCW seems to be high as it was conducted in N₂-HCW. This would be considered that [H⁺] in CO₂-HCW is higher than those in N₂-HCW. Accordingly, we estimated [H⁺] in each reaction fields. [H⁺] formed in each reaction fields were listed in Table 1. [H⁺] ranged from 2.71×10^{-4} to 3.87×10^{-4} mol/kg formed in CO₂-HCW ranged from 1.53×10^{-6} to 1.85×10^{-6} mol/kg are approximately 100-fold higher than that of created in N₂-HCW. The Henry's constant (K_H) of carbon dioxide which represents solubility of carbon dioxide into water behaves the convex downward and reaches the minimum at temperature around 150°C (Hunter and Savage, 2008). For this reason, in CO₂-HCW at 100–150 °C, the solubility of carbon dioxide into water slightly decrease and [H⁺] results in lower with temperature increase. On the other hands, in N₂-HCW at 150–170°C, [H⁺] increase with temperature due to K_w also increase with temperature. [H⁺] in N₂-HCW cannot reach to the amount of hydronium ions in CO₂-HCW because the maximum of K_w is about -11 [$\log K_w$ (mol/kg)²], which means the maximum of [H⁺] is about 3.16×10^{-6} . The intramolecular dehydration of fructose occurred to proceed at 160–170°C.

The temperature dependence of the absorbance of inulin hydrolyzate at 284 nm was shown in Fig. 3. In N₂-HCW, the absorbance at 284 nm representing the presence of 5-HMF increase with temperature (Fig. 4 (b)), espe-

Table 1 The hydronium ion concentration at each experiments (T_p : preset temperature, T_f : reached temperature, P_i : preset pressure, P_f : reached pressure).

	T_p [°C]	T_f [°C]	P_i [MPa]	P_f [MPa]	[H ⁺] [mol/kg]
CO ₂ -HCW	100	99.1	3.00	3.40	3.87×10^{-4}
	110	110.0	3.00	3.71	3.55×10^{-4}
	130	130.2	3.00	3.68	3.23×10^{-4}
	150	148.1	3.00	4.29	2.71×10^{-4}
N ₂ -HCW	150	149.0	3.00	4.00	1.53×10^{-6}
	155	153.2	3.00	4.09	1.60×10^{-6}
	160	159.3	3.00	4.20	1.70×10^{-6}
	165	163.5	3.00	4.23	1.78×10^{-6}
	170	167.8	3.00	4.23	1.85×10^{-6}

cially converting inulin into DFAs needs to be treated at higher temperature range (160–170°C). On the other hands, in CO₂-HCW, the converting inulin into DFAs was attained at 130–150°C and the absorbance at 284 nm is relatively low. HMF is produced by dehydration action of the hot compressed water, i.e., inulin is hydrolyzed to fructose, and then converted into HMF via intramolecular dehydration of fructose (Fig. 4). The low temperature (130–150 °C) and high [H⁺] enable to produce DFAs with suppressing generation of 5-HMF in CO₂-HCW.

In conclusion, we conducted the hydrolysis of inulin with CO₂-HCW (100–150°C, 3.0–5.0 MPa) in a batch-type reactor. The results of ESI-MS and UV-Vis of the inulin hydrolysate showed that inulin was hydrolyzed to FOSs, fructose, HMF and DFAs. The hydrolysis of inulin proceeded at higher temperature and the components of the inulin hydrolysate varied depending on the preparation temperature. The major products of inulin hydrolysate prepared with CO₂-HCW at 130–150°C were DFAs and the generation of HMF were significantly suppressed. This is

Fig. 4 The temperature dependence of the absorbance at 284 nm. (a) CO₂-HCW, (b) N₂-HCW.

Scheme 1 The reaction mechanism of hydrolysis of inulin with (a) CO₂-HCW and (b) N₂-HCW.

owing to the characteristic of CO₂-HCW regarding to the high [H⁺] (Scheme 1). The estimation of [H⁺] and internal energy of each reaction fields revealed that [H⁺] created with CO₂-HCW at temperatures from 130 to 150°C were 100-fold higher than the ones created with N₂-HCW at 150°C. The current study demonstrated that CO₂-HCW is one of option of converting inulin into DFAs while suppressing generation of HMF.

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