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Shochu Koji Microstructure and Starch Structure during Preparation

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Abstract: In this study, we investigated the changes in composition, microstructure, and starch molecular structure of shochu koji during preparation. We observed that the gelatinized and outer part of starch was decomposed in priority during the early and middle preparation stages. The gap between the starch granules increased with the delayed time. Finally, the koji microstructure became spongy. Shochu koji mold produced two α-amylases in different expression manners. Acid-labile α-amylase was produced in the early and middle preparation stages. Acid-stable α-amylase and saccharification power were produced in the middle and late stages. Throughout the koji preparation, reducing sugars content reached approximately 13−20 % of the total sugar content, with glucose representing over 70 % of the reducing sugars. α-Glucan fragments with C chains of degree of polymerization (DP) 4−73 were observed in the early and middle stages (<23 h), indicating the degradation of amylopectin at long B chains. In the latter stage, the amount of C chains of DP 6−30 decreased, while the longer C chains (DP 30<) did not change. These results showed that acid-labile α-amylase, acid-stable α-amylase, and saccharification enzymes including glucoamylase and α-glucosidase work preferentially on the amorphous regions of starch granules, and cooperative action of these enzymes during koji preparation contributes to the formation of the observed microstructure. Our study is the first report on the decomposition schemes of starch and the microstructure forming process in shochu koji.

Key words: shochu koji, starch structure, degradation, glucoamylase, α-amylase, koji preparation

INTRODUCTION

Shochu is a traditional Japanese spirit with a 500-year history. Shochu is recognized as a national alcoholic drink of Japan, which is referred to as "Kokushu" in Japanese. According to the annual report of the national tax administration agency, 413 kL shochu was consumed in 2019 (https://www.nta.go.jp/taxes/sake/tokei/kazeikankei2019/pdf/31-03.pdf). The consumption volume is almost the same as that of sake (451 kL). Thus, shochu is one of the important products of Japan.

Although shochu could be produced from several plant materials such as sweet potato, rice, and barley, "koji" is a common material in all shochu types. Koji refers to a solid-state koji mold culture on steamed cereals grains such as rice. Koji contains various enzymes produced by the koji mold during growth, thereby providing an enzyme source, similar to malt for beer, to decompose foodstuffs such as starch, proteins, and lipids. Koji molds used in Japan mostly belong to *Aspergillus oryzae*, *Aspergillus luchuensis* mut. *kawachii*, and *Aspergillus luchuensis* (yellow, white, and black koji molds, respectively). Accordingly, koji prepared using each koji mold type is named yellow, white, and black koji, respectively. Yellow koji is used for producing sake (rice wine), shoyu (soy sauce), miso (fermented soy paste), and shochu, while white and black kojis are exclusively used for shochu preparation, and they are thus also referred to as shochu koji. The clear difference between the two mold groups is that white and black koji molds secrete a relevant amount of citric acid.[1)] Therefore, shochu koji displays high citric acid concentration, lowering the mash pH and contributing to contamination prevention during alcohol fermentation.

A scheme of shochu koji preparation typically takes approximately 42 h (Fig. 1). It was described as follows. In the initial step, a seed culture is inoculated onto steamed rice. This inoculation step is called "Tane-tsuke". The inoculated rice is then incubated in the mass for 19 h in an incubation room at around 35 °C. Following incubation, the temperature of koji rise to 39−40 °C after incubation and the mass is mixed well by hand with air to lower it to approximately 33−35 °C, ensuring uniform culture conditions. The well-mixed koji is divided into 1 kg portions, and each portion is placed in a wooden box. This process is called "Mori" (dividing). The

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divided koji was further incubated at approximately 35 ˚C. The temperature of koji begins to rise again with the growth of koji mold. The incubation room temperature is controlled to ensure that koji temperature would not exceed 40 ˚C. After 4 h (23 h after inoculation), koji temperature reaches 39−40 ˚C, and then koji is mixed well again for cooling to 35 ˚C. This second mixing step is called "Naka-shigoto" (middle work). The incubation process continues, and when the temperature reaches 39−40 ˚C again, approximately 4 h later (27 h after the inoculation), it is mixed once more. This third mixing step is called "Shimai-shigoto" (latter work). After Shimai-shigoto, the incubation continues, but the room temperature is slightly lowered at this point to maintain koji temperature at approximately 35 ˚C. The controlled temperature adjustment after Shimai-shigoto is essential for promoting the production of citric acid by koji mold. This temperature control is not present in the case of yellow koji preparation. After an additional 15-h incubation, koji is removed from the incubation room, and the preparation is complete. This final step is called "De-koji" (final koji product).

White and black koji molds produce two types of α-amylases: an acid-labile and an acid-stable α-amylase.[2)3)4)] These α-amylases exhibit different expression patterns during koji preparation.[5)] The expression of acid-labile α-amylase keeps increasing from inoculation until Shimai-shigoto. During the incubation period at lowered temperature after Shimai-shigoto, the koji mold produces a significant amount of citric acid. Concomitantly, the acid-labile α-amylase activity decreases, primarily due to the denaturation of acid-labile α-amylase at the low pH derived from citric acid. In contrast, acid-stable α-amylase activity increases after Shimai-shigoto. In the final koji detectable α-amylase activity is almost exclusively due to acid-stable enzyme. Therefore, the acid-stable α-amylase activity could mainly be observed during alcohol fermentation in the shochu moromi-mash. On the contrary, acid-labile α-amylase activity is generally considered irrelevant in shochu production.

In our previous study, however, we revealed that acid-labile α-amylase plays an important role in starch decomposition in koji using a laboratory strain of white koji mold.[5)] The sponge-koji microstructure is formed as a result of the degradation of starch granules in rice grains, and it is attributed to the cooperative successive actions of acid-labile and acid-stable α-amylases. The koji microstructure reportedly plays a role in accelerating alcohol fermentation speed at the early stage and in flavor formation.[5)] The koji microstructure is presumed to affect the water absorption and swelling speed of starch granules in the koji, eventually accelerating the starch degradation and alcohol fermentation. The koji microstructure is expected to be related to the granular and molecular structure of starch, while the starch structure in koji and the starch degradation scheme during koji preparation are yet to be investigated.

In this study, using a commercial koji strain for shochu making, we analyzed the changes in amylolytic activities, acidity, and molecular structure of starch during shochu koji preparation. Here, we report new insights into fermentation control for shochu manufacturing, including confirmation of the importance of acid-labile α-amylase in koji preparation with a commercial strain.

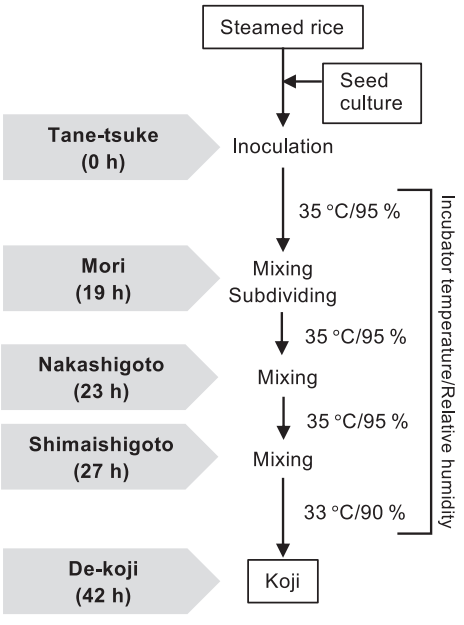

Fig. 1. Shochu koji preparation.

MATERIALS AND METHODS

Materials, chemicals, and strains. Polished rice (Japonica rice) was purchased from Hombo Shoten Co., Ltd. (Kagoshima, Japan). White koji mold seed culture was purchased from Kawachi Gen-ichiro Shoten (Kagoshima, Japan). The Kagoshima-5 yeast strain was used in this study, supplied by the Kagoshima Prefectural Brewing Association.

Koji preparation. A quantity of 600 g of polished rice was soaked in water for 1 h and drained off for an additional 1 h. The soaked rice was then steamed for 1 h and cooled to 45 ˚C before inoculation. The seed culture of shochu koji mold 6 g was inoculated into the steamed rice and mixed thoroughly. The inoculated rice was incubated under a specific schedule of temperature and humidity over 42 h: first, a 35−38 ˚C linear gradient for 19 h, with relative humidity (RH) of 95 %, followed by 38 ˚C for 8 h, at RH of 95 %, and finally 35 ˚C for 15 h, at RH of 90 %. In addition, the koji was mixed at 19, 23, 27, and 42 h, which are the mixing points of industrial koji preparation to cool the koji and homogenize growing conditions. Koji samples of 100 g were sampled just after inoculation and at 19, 23, 27, and 42 h.

Scanning electron microscopy (SEM). The grain samples were mounted on an aluminum stub using double-sided adhesive tape, with the hand-cut section oriented upwards. The samples were coated using a quick auto coater (JFC-1500, JEOL Ltd., Tokyo, Japan) with a platinum layer under a vacuum. The coated samples were observed using an SEM (Miniscope TM3000, Hitachi High-Technologies Co., Tokyo, Japan).

Moisture content. The moisture content of the samples was analyzed using an infrared moisture analyzer (FD-720, Kett Electric Laboratory Co. Ltd., Tokyo, Japan). Five grams of the samples were placed on an aluminum dish, and the instrument calculated the sample moisture contents automatically.

Reducing sugar and glucose content. Koji samples were freeze-dried and ground to powder. Powdered samples of 2

g were added to 10 mL of deionized water and maintained at room temperature for 3 h. Next, the suspension was centrifuged at 890 × *g* and 4°C for 10 min, and the supernatant was subjected to analysis. The glucose content was measured using high-performance liquid chromatography[6] and the reducing sugar content was determined using the Somogyi-Nelson assay.[7][8] Reducing sugar and glucose content per gram of anhydrous koji were calculated by using the moisture content in the powder.

Total sugar analysis. The total sugar content was measured via the Somogyi method after acid hydrolysis treatment.[9][10] Koji samples were freeze-dried and ground to powder. One gram of the powdered sample was added to 100 mL of deionized water and 10 mL of 7.7 M HCl and heated for 2.5 h in boiling water. After cooling, the samples were neutralized using sodium hydroxide solution and completed to a volume of 200 mL. The samples were filtrated using a paper filter. The filtrate was analyzed using the Somogyi method. The Somogyi method involved the following procedure: 10 mL of Somogyi solution A (90 g of Rochelle salt, 225 g of Na_3PO_4•$12H_2O$, 30 g of $CuSO_4$•$5H_2O$, and 3.5 g of KIO_3 dissolved in 1,000 mL deionized water) was mixed with a sample 20 mL in an Erlenmeyer flask. The mixture was heated until it reached boiling point, and then it was maintained at a steady boil for precisely 3 min. Subsequently, 10 mL of solution B (90 g of potassium oxalate monohydrate and 40 g of potassium iodide dissolved in 1,000 mL deionized water) was added and thoroughly mixed, followed by the addition of 10 mL of solution C (2 M sulfuric acid). The resulting mixture was promptly titrated with solution D. A 1 % (w/w) starch solution was used as the indicator. The amount of reducing sugars equivalent to glucose was determined by titration, and the total sugar content per gram of anhydrous koji was calculated based on reducing sugar content and the moisture content in the powder.

α-Amylase assay. Two grams of koji sample were added to 10 mL of 100 mM sodium acetate buffer (pH 5.0) containing 0.5 % (w/v) NaCl, maintained at 4°C overnight. Thereafter, the suspension was centrifuged at 890 × *g* and 4°C for 10 min, and the supernatant was analyzed. The total α-amylase activity was determined using an α-amylase assay kit (Kikkoman Biochemifa Co., Tokyo, Japan). The acid-stable α-amylase activity was assayed following.[2] The crude extract was then diluted with a 50 mM glycine-HCl buffer (pH 3.0) and incubated at 30 °C for 1 h. Next, this solution was diluted with a 100 mM sodium acetate buffer (pH 5.0). The α-amylase activity was assayed similarly. The acid-labile α-amylase activity was estimated from the difference between the total and acid-stable α-amylase activities. One unit of α-amylase was defined as the amount of enzyme required to release 1 μmol of 2-chloro-4-nitrophenol from 2-chloro-4-nitrophenyl 6-azide-6-deoxy-β-maltopentaoside per min at 37 °C. α-Amylase activity per gram of anhydrous-koji were calculated by using the moisture content in the powder.

Saccharification power assay. The saccharification power was determined using a saccharification power measuring kit (Kikkoman Biochemifa Co.). One unit of saccharification power was defined as the amount of enzyme required to release 1 μmol of 2-chloro-4-nirtophenol from 4-nitro-phenyl-β-maltoside per min at 37 °C. Saccharification power per gram of anhydrous koji were calculated by using the moisture content in the powder.

Koji acidity. Total acidity values were determined following the official methods of the National Tax Administration Agency, Japan (Brewing Society of Japan, 2006, pp. 221−223). Ten grams of koji were added to 50 mL of deionized water. Ten milliliters of extract was titrated against 0.1 M sodium hydroxide, with the indicator containing bromothymol blue and neutral red, until the solution turned light green. Total acidity was represented in the titration volume of 0.1 M sodium hydroxide.

Chain-length distributions of α-glucans in koji. Chain-length distributions of debranched α-glucans in koji samples were analyzed by high-performance size-exclusion chromatography (HPSEC) with pre-column fluorescent labeling of reducing residues according to the previous study.[11] Koji samples (ca. 100 mg) at 19-, 23-, 27-, and 42-h incubation were freeze-dried and ground to powder. Five mg of the powdered sample were subjected to debranching with isoamylase followed by labeling with 2-aminopyridine as described[11] with minor modifications as follows. The heat-gelatinized powdered-koji sample for isoamylolysis contained insoluble materials. They were removed after isoamylolysis by centrifugation (Model 2700 with an RS-240 swing rotor, Kubota Corp. Co. Ltd., Tokyo, Japan) at 3,000 rpm for 5 min at ambient temp., and only the supernatant was lyophilized and subjected to the subsequent labeling procedure. The labeled sample was analyzed with a HPSEC system equipped with a differential refractive-index detector and a fluorescence detector for monitoring a weight- or a molar-based distribution, respectively. The resulting distribution represents that of whole unit chains, including amylose as well as small oligo-saccharides that were produced by endogenous amylolytic activities during koji preparation.

Chain-length distributions of the main chain (C chain) were analyzed as described[11] for both the powdered koji sample and its ethanol (75 %) precipitable fraction. The 75 %-EtOH precipitable fraction was obtained by following the labeling procedure for the C chain of amylopectin,[11] but the addition of labeling reagents was omitted. Briefly, 6 mg of koji powder was heat-gelatinized in 0.2 mL of 50 % DMSO and diluted with 0.3 mL of deionized water, and then 3 vol. of EtOH and NaCl (final conc. 10 mM) were added, and the mixture was cooled on ice for 1 h. The precipitate was collected by centrifugation (3,000 rpm for 10 min at 4 °C) and was washed with 1 mL of cold 75 % EtOH–10 mM NaCl. The C chain profile of 75 %-EtOH precipitable fraction essentially represents the main chains of amylose and amylopectin. The C chain profile for the ethanol non-precipitable fraction was obtained by subtracting the C chain profile of 75 %-EtOH precipitable fraction from that of the koji sample without prior ethanol precipitation.

RESULTS

Changes in the physical structure of shochu koji during preparation.

We observed shochu koji property changes, such as the appearance, enzymatic activity, and saccharide composition during preparation. We monitored the process points of

Tane-tsuke (starter culture inoculation, 0 h after inoculation), Mori (first mixing, 19 h after inoculation), Naka-shigoto (second mixing, 23 h after inoculation), Shimai-shigoto (third mixing, 27 h after inoculation), and De-koji (final product, 42 h after inoculation) (Fig. 1).

Changes in appearance and internal structure of rice grains during koji preparation were observed (Fig. 2) and compared with those of raw rice (Figs. 2a, 2g, 2m) and the inoculated rice (0-h incubation). The inoculated rice was observed for gelatinization by steam heating (Figs. 2b, 2h, 2n). In the rice sample incubated for 19 h, a white part appeared on the surface of the grains, indicating the growth of the koji mold (Fig. 2c). The inside of the grain exhibited a slightly uneven-rounded appearance (Figs. 2i, 2o). The rice samples incubated for 23 and 27 h visibly contained white parts (Figs. 2d, 2e). The uneven-rounded appearance inside the grain, considered the starch granule, could also be observed more clearly (Figs. 2j, 2p, 2k, 2q). The size of this structure appeared to be 2−10 μm. The rice starch granule size is 2−10 μm.[12] Therefore, we concluded that the observed structure should be the rice starch granule and inferred that the gelatinized softer parts around the granules would be preferentially decomposed by the enzyme, resulting in the appearance like sponge in the koji. The surface of the rice sample incubated for 42 h (koji) was completely covered by the koji mold mycelia (Fig. 2f). The uneven-rounded structure disappeared (Figs. 2l, 2r), the surface structure became rugged, and the granule appeared to be broken down.

Moisture content, enzymatic activity, and acidity changes in shochu koji during preparation.

The steamed rice moisture content generally varies between 36−38 % (w/w). We observed a slight decrement of moisture content over time (Fig. 3A). The moisture content of the final koji product was almost the same as that of industrial-produced koji. The total sugar content in the dry sample decreased over time (Fig. 3B). Koji acidity is among the generally analyzed characteristics of shochu koji also measured in the shochu industry. We observed that koji acidity increased with time (Fig. 3C). In particular, acidity increased remarkably 27 h after inoculation. The sugar content was potentially consumed for the growth and citric acid production of the mold. The soluble reducing sugar and glucose contents increased at the time point of 19 h (Fig. 3B). These contents gradually increased after 19 h until 27 h and slightly decreased at 42 h. The reducing sugar content reached approximately 13 % of the total sugar content at 19 h and 20 % of that at 27 h and 42 h, the glucose contents accounting for a high proportion in them (71 % and approximately 90 % of reducing sugar at 19 h and 23−42 h, respectively). These results indicated that the released oligosaccharide in koji was quickly broken down into glucose.

The enzymatic activities of the two α-amylases were detected by different manners (Fig. 3D). The activity of acid-labile α-amylase increased until 23 h, but it decreased at 27 h and could not be detected in koji at 42 h. In a previous study, koji prepared using a laboratory koji mold strain exhibited low acid-labile α-amylase activity at approximately a quarter of the acid-stable α-amylase activity.[5] The acidity of koji prepared in this study was higher than that of the aforementioned koji. It has been reported that 90 % of the acid-labile and acid-stable amylases were stable in the pH ranges of 4.5−9.5 and 2.0−6.5, respectively.[13] This result indicated that acid-labile α-amylase was completely denatured while acid-stable α-amylase activity increased gradually throughout koji making. In this study, saccharification power was measured as total activity of glucoamylase and α-glucosidase. Saccharification power also increased throughout koji making. Although enzymatic activities increased or were maintained at the same level throughout koji making, reducing sugar and glucose contents slightly decreased from 27 h to 42 h, a part of the glucose content would thus be potentially used for growth and citric acid production.

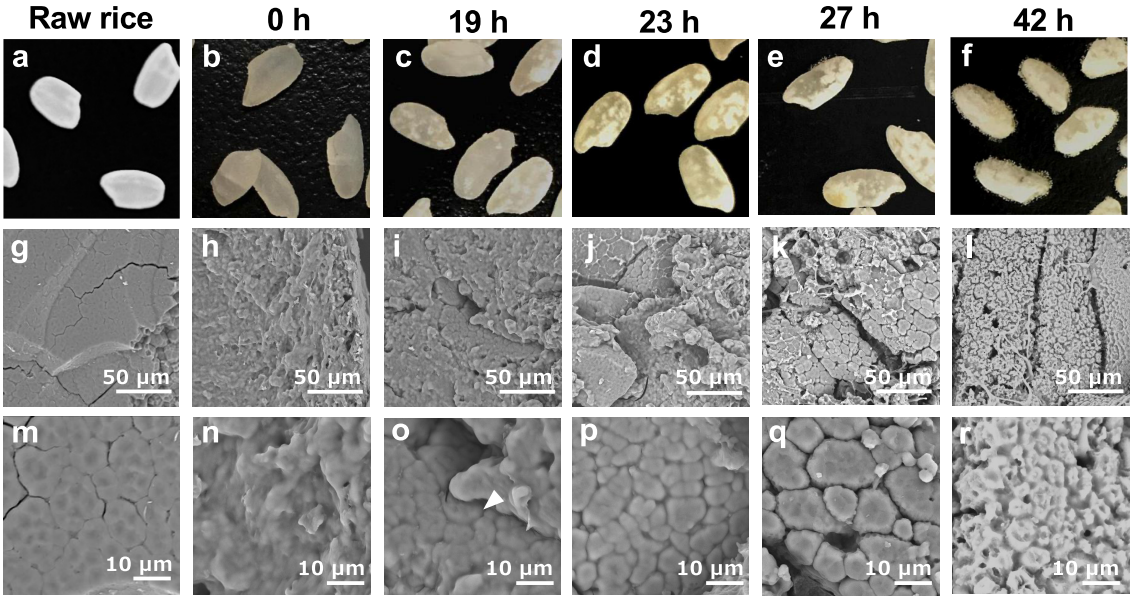

Fig. 2. Koji samples at the different process points of koji preparation.

Photographs (a–f) and Scanning electron microscope micrographs (g–r). Raw rice (a, g, and m) and koji samples observed at the process points of 0 (Tane-tsuke; b, h, and n), 19 h (Mori; c, i, and o), 23 (Naka-shigoto; d, j, and p), 27 (Shimai-shigoto; e, k, and q), and 42 (De-koji; f, l, and r) hours.

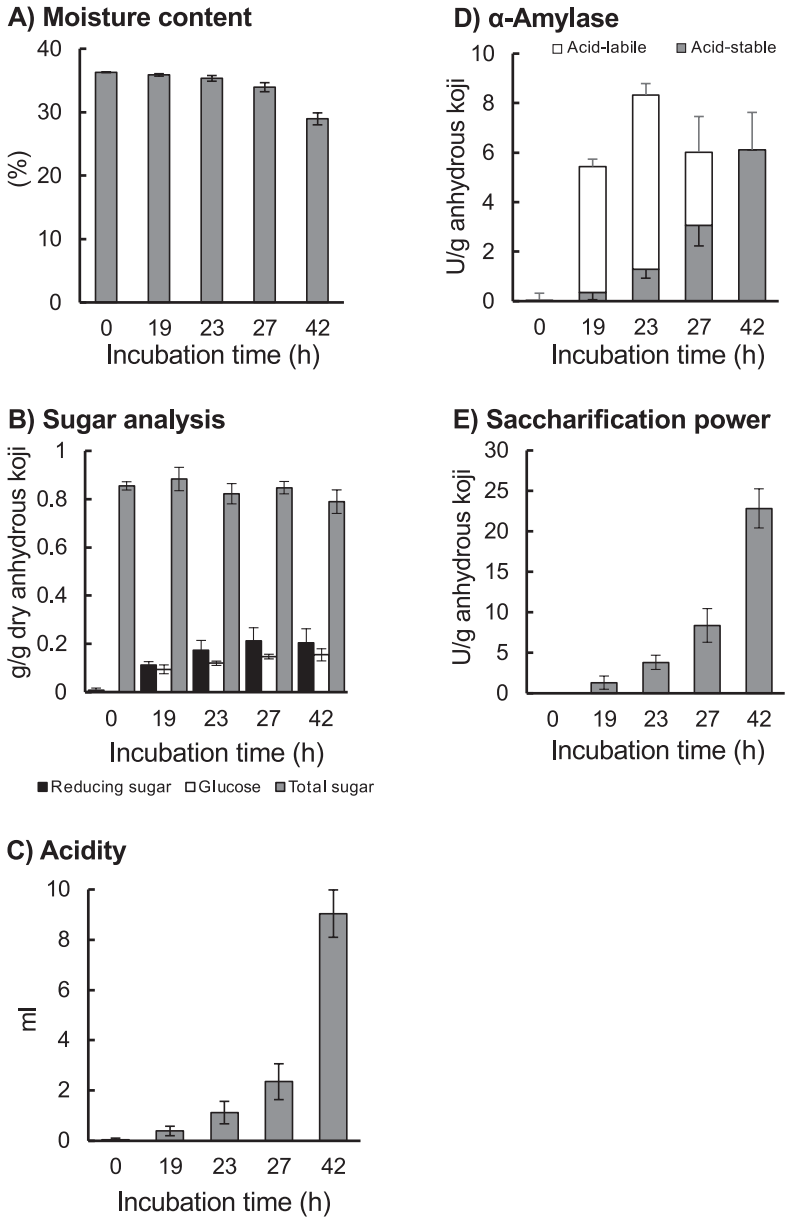

Fig. 3. Koji moisture and sugar contents, enzymatic activity, and acidity.
Koji samples were analyzed at the preparation process points of 0, 19, 23, 27, and 42 h.

Starch structure changes in shochu koji during preparation.

To understand the starch decomposition that occurred during koji preparation, we characterized changes in the structure of α-glucan components of rice starch granules of koji samples. In Fig. 4, a typical result (koji at 23 h) of chain-length distribution of rice starch in the koji sample was compared with that of rice starch from grains used in koji preparation. Regardless of the preparation stages, all the koji samples showed very similar chain-length distributions by both a weight- and a molar-basis to those of original rice starch (koji sample at 0 h, see Table 1). The chain-length distribution was divided into three fractions in the order of elution: apparent amylose, B2+B3, and A+B1 fractions, as shown in Fig. 4A. The latter two fractions are composed of the unit chains of amylopectin. The designation of the fractions was according to Hizukuri.[14] The amount of apparent amylose (apparent amylose content) decreased slightly only at the late stage (42 h) of koji preparation (Table 1), suggesting that amylose remained intact until koji preparation fully progressed. In the case of amylopectin, no obvious changes in the amount and the ratios of unit-chain fractions of both long, cluster-connecting chains (B2+B3) and short, cluster-composing chains (A+B1). The absence of changes in chain-length distributions indicates that the amylolytic degradation of starch in rice grains occurred only to a limited extent, while microscopic observation (Fig. 2) revealed vigorous changes in the appearance of starch granules in koji samples during their preparation. However, hydrolysis of unit chains definitely occurred as indicated by the occurrence of short chains of DP less than 6. Compared to the control (koji at 0 h), chains of DP less than 6 were newly formed, and fluorescence intensity of DP 6−7 peaks increased slightly in the koji at 23 h (Fig. 4).

In addition to the unit chains, we focused on the main chain of the α-glucan molecules. The main chain, also referred to as C chain, is the only constituent chain to retain its reducing residue. Also, the amount of C chain is expected to increase due to the action of glycoside hydrolases such as α-amylase during koji preparation. In order to simplify the

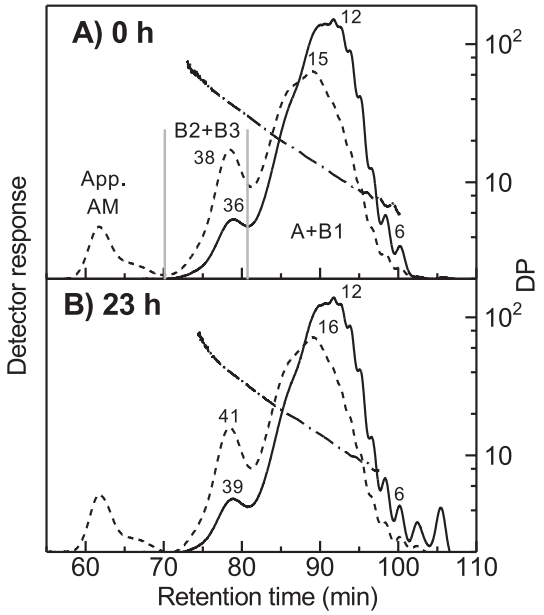

Fig. 4. Comparison of the chain-length distributions of rice starches in koji samples at different stages of koji preparation.

(A) Koji sample at 0 h. (B) Koji sample at 23 h. Solid and dashed lines show fluorescence and refractive index detector response, respectively. Dash-dot-dashed line indicates DP at given elution positions. Numbers indicate peak-DP. The distributions were divided into three fractions (apparent amylose (App. AM), and long (B2+B3) and short (A+B1) unit-chains of amylopectin as shown in panel A.

Table 1. Chain-length distributions of rice starches from different stages of koji preparation.

Sample	Amount (%)			Ratio,
	App. AM	B2+B3	A+B1	(A+B1)/(B2+B3)
by weight				
0 h	7.4	19.2	73.4	3.8
19 h	8.1	19.7	72.2	3.7
23 h	7.4	17.7	74.8	4.2
27 h	7.2	20.7	72.2	3.5
42 h	6.0	19.9	74.1	3.7
by mole				
0 h	-	8.2	91.8	11
19 h	-	7.2	92.8	13
23 h	-	7.0	93.0	13
27 h	-	7.5	92.5	12
42 h	-	7.6	92.4	12

Apparent amylose fraction includes amylose and extra-long chains of amylopectin. This fraction was not determined on a molar-basis.

interpretation of the experimental results, the powdered koji sample was first fractionated by ethanol precipitation to isolate the EtOH-precipitable fraction, which comprised high-molecular-weight α-glucans. Secondly, the koji sample with or without prior EtOH precipitation was subjected to labeling with a fluorophore at their reducing end. The labeled product was debranched with isoamylase, and then subjected to chain-length distribution analyses of C chains. Due to practical difficulties, EtOH non-precipitable fraction was not directly analyzed, in which low-molecular-weight α-glucans and malto-oligosaccharides are presumed to be present. Instead, a fluorescence profile of an EtOH precipitable fraction was subtracted from that of koji sample without EtOH precipitation. The subtraction was performed after normalization of total peak area by RI detection (Fig. 5A). The differential profiles so obtained were regarded as C chain distribution of EtOH non-precipitable fractions (Fig. 5C).

For the EtOH precipitable fraction (Fig. 5B), the C chain profiles can be divided into three regions at retention times of 60−70 min, 70−95 min, and >95 min. The two peaks in the first region are assigned essentially to amylose. Changes in peak height and shape by the different stages of koji preparation were relatively small, being consistent with the finding on the scarce degradation of amylose (Table 1). Changes in C chain profiles were most prominent in the second region, in which the chains of DP ca. 10−80 were eluted. The control sample (koji at 0 h) showed the same feature of C chain profile of rice amylopectin as those reported previously.[11)15)] Two peaks at DP 73 and 36 increased progressively, especially in the early stage of 0 to 19 h, and they remained as a peak in koji at 42 h. On the contrary, chains of DP <24 increased up to 23 h but then decreased rapidly afterward. In the third region, short chains of DP 4−10 changed over time in a similar manner to those of chains of DP <24.

The C chain profiles also provided a clue to the changes in the molecular size of amylopectins during koji preparation. The ratio of refractive index/fluorescence detector responses (RI/F ratio) is proportional to the number-average degree of polymerization, which is calculated as the ratio of total/reducing glucosyl residues after the determination of residues of each category by suitable methods. The RI/F ratio was determined from the corresponding peak area between retention time of 74 and 112 min, and amylose was excluded from the calculation. Note that the RI profiles of koji samples are not presented in the Fig. 5B to avoid complexation of the figure, but the actual data were collected and they were almost identical to each other, as shown in the two examples in Fig. 4. Relative values of the RI/F ratio of each koji sample were as follows: 0 h, 1.00; 19 h, 0.38; 23 h, 0.30; 27 h, 0.56; 42 h, 0.61, indicating that amylopectin in rice starch became smaller, 1/3 to 1/2 in size of original amylopectin. Assuming that the hydrolysis occurred at only one α-1,4 glucosidic linkage in each molecule of amylopectin and both of the resulting products were EtOH precipitable, the relative ratio would decrease to 0.5. Likewise, hydrolysis of two linkages would result in a relative ratio of 0.33. Therefore, the observed RI/F ratios for the koji samples (0.30−0.61) suggest that the endo-wise hydrolysis onto amylopectin molecules occurs only at a few sites per molecule, leaving the product still large enough to be precipitated with EtOH. The increase in the ratio from 0.30 to 0.61 at the middle to late stages of koji preparation does not necessarily means an increase in the size of amylopectin because the composition of the 75 %-EtOH precipitate would be determined by the different state of hydrolysis at different stages of koji preparation and the composition, in turn, would have affected to the measured value of RI/F ratio.

The EtOH non-precipitable fraction (Fig. 5C) is mainly composed of short chains with chains of DP <24 (retention time around 85 min or longer) being dominant, and again their changes over time were essentially the same as those of the chains of DP <24 of the EtOH precipitable fraction. A notable exception was that the long C chains, which appeared in a retention time of 75 min or shorter, were only detected in the koji at 42 h. Such long C chains arose most likely from amylose, indicating that amylose degradation occurred only in the very late stage of koji preparation.

DISCUSSION

It has been reported that the flavor properties of shochu are partly influenced by koji mold used in the production. It is suggested that volatile compounds, which are the secondary metabolites of koji mold, or volatile compounds contained in the plant ingredients and released by the enzymes of koji mold, transit directly from the fermentation mash to shochu.[16)17)] Furthermore, it has been observed that the microstructure of koji plays a crucial role in shaping the flavor of shochu.[5)] Therefore, comprehending the formation process of microstructure in koji holds paramount significance, not only from a scientific standpoint but also for its industrial applications in the precise control of flavor attributes in fermentation products.

To date, a detailed investigation into the digestion manner of starch by α-amylase from *Aspergillus luchuensis* has not yet been reported. Furthermore, there are limited studies available that provide a comprehensive understanding of starch digestion by α-amylase derived from *Aspergillus* sp. and other microbes. Meanwhile, some studies have conducted the application of α-amylase to reduce the firming rate of bread crumbs during storage.[18)] Amylolitic modification of starch has been reported to alter the retrogradation behavior.[19)] Leman *et al.* conducted a comprehensive investigation on the structural changes in starch hydrolyzed by Taka-amylase from *Aspergillus oryzae*.[20)] They observed that Taka-amylase

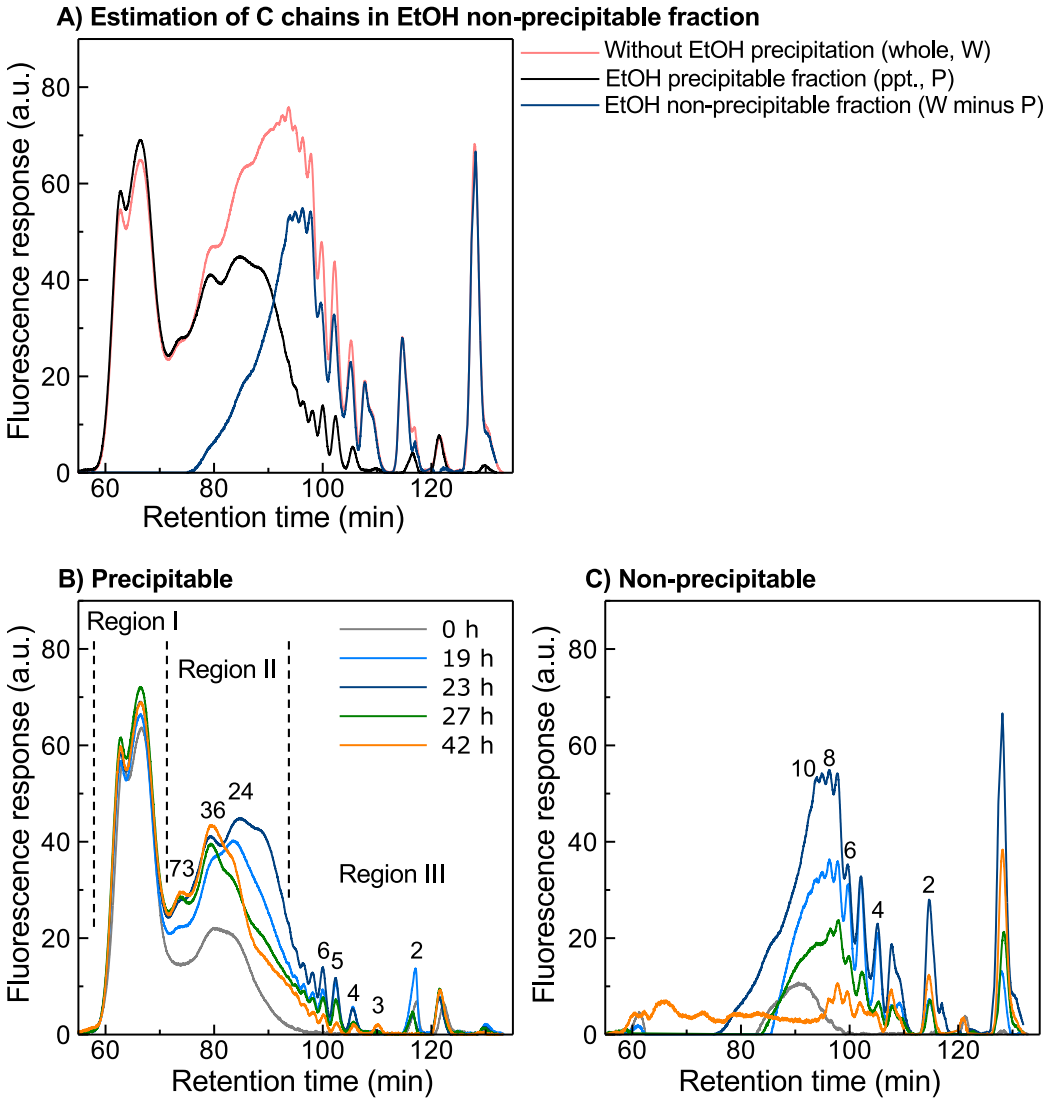

Fig. 5. Degradation of amylose and amylopectin, and generation of small oligosaccharides during koji preparation as judged by the C (main) chain profiles.

(A) An example (koji sample at 23 h) of the estimation of C chains in the EtOH non-precipitable fraction. (B) The C chains of EtOH precipitable fractions (mainly those of amylose and amylopectin). (C) The C chains of the EtOH non-precipitable fractions (mainly those of fragments or oligosaccharides generated by endogenous amylolysis). Numbers above C-chain profiles indicate DP at each elution position. The gain setting of the detector was 100-fold higher than that for analyses shown in Fig. 4.

treatment caused slight differences in the chain-length distribution of amylopectin, particularly a slight increase in long B chains (B2, B3, and B4). Leman *et al*. performed amylolysis of purified amylopectin using commercial Taka-amylase and the Rapid Visco Analyser apparatus.[20] Although the conditions for amylolysis were quite different between this study and the previous study by Leman *et al*.,[20] changes in the chain-length distribution of amylopectin and the consequent presumption for the action of acid-labile α-amylase on starch granules agreed well with each other, that is, the enzyme attack preferentially on the long B chains of amylopectin. The primary structure of Taka-amylase from *Aspergillus oryzae* shows approximately 100 % and 74 % identity with acid-labile and acid-stable α-amylases, respectively, from *Aspergillus luchuensis* mut. *kawachii* IFO4308. The enzymatic properties of acid-labile α-amylase from *A. luchuensis* are almost identical to those of Taka-amylase.[21] Thus, Taka-amylase can be considered a homolog of acid-labile α-amylase. Thus, it is strongly suggested that the acid-labile α-amylase contributes significantly to starch degradation during food production in neutral as well as in acidic pH.

Glucoamylase and α-amylase in the white and black koji molds have been found to possess the ability to degrade raw starch.[22][23] Previous studies have observed that the appearance of granular surface of raw rice remain unaltered when exposed to glucoamylase derived from black koji mold alone. However, when starch granules are exposed to α-amylase from black koji mold, the distinctive holes are observed on the granule surface.[23] When glucoamylase and α-amylase are employed simultaneously, the number and diameter of the holes on the granule surface are increased significantly. Notably, the resulting appearance closely resembles that of the final koji sample in this study. These findings strongly indicate that the formation of final structure on starch granules is attributable to an effect exerted by glucoamylase and α-amylase.

Our conclusion on the starch degradation scheme in the koji samples is as follows, and possible sites of amylolysis are shown in Fig. 6. From Tane-tsuke to Naka-shigoto, koji mold produces preferentially the acid-labile α-amylase, and the hydrolysis of B2 or longer chains in the starch molecules occurs. As a result of the amylolysis, the ethanol-precipitable, high-molecular-weight products are produced (Fig. 6A). After Naka-shigoto, the mash-pH has decreased gradually because of accumulation of citric acids, and then the production of the acid-stable α-amylase by koji mold is triggered by the low pH. The two α-amylases now digest the glucans cooperatively. The glucan fragments with C chains having DP 6−30 have been generated by Naka-shigoto. The fragments are further hydrolyzed at B1 and A chains, generating short, linear oligosaccharides (Fig. 6B). After Shimai-shigoto, the resulting oligosaccharides are digested completely to glucose by the simultaneous actions of acid-stable α-amylase and glucoamylase. The glucose now serves as a carbon source for growth and citric acid produc-

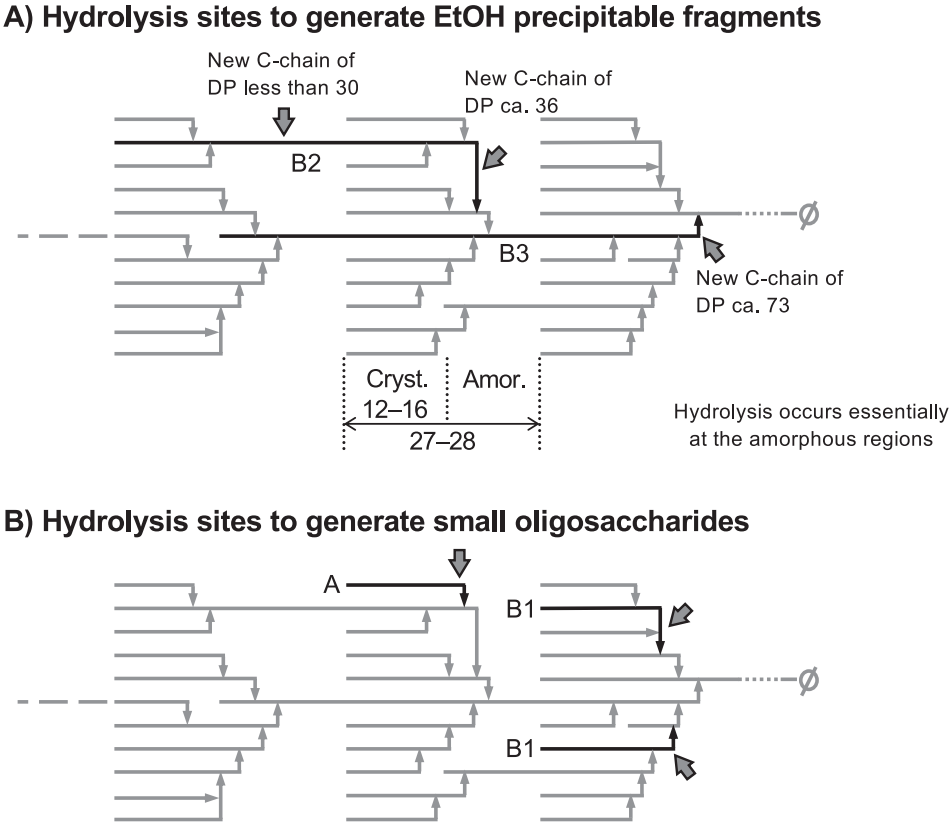

Fig. 6. The starch decomposition models of shochu koji.

(A) Possible hydrolysis sites of B2- or longer chains to generate EtOH-precipitable fragments with new C-chains. Arrows indicate sites where the fragments with C-chains of the designated DP (<30, ca. 36, and ca. 73), which were observed as characteristic peaks in Fig. 5B. (B) Possible hydrolysis sites of A- or B1-chain to produce small, linear, or slightly branched oligosaccharides. Those products were detected in the EtOH non-precipitable fractions (Fig. 5B). The fragments proposed in (A) are also susceptible to the hydrolysis proposed in (B).

tion of koji mold. In this study, we propose a process that forms the microstructure in koji and a starch degradation scheme behind the observed sponge-like structure. Early stage of alcohol fermentation in the mash is significantly complex. Our results would contribute to the common fundamental knowledge in the related fields to understand alcohol fermentation during shochu production.

CONFLICTS OF INTEREST

The authors declare that they have no conflict of interest.

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