Regular paper

Kinetics of structural changes in starch retrogradation by SANS/FTIR–ATR

Kinetics of structural changes in starch retrogradation observed by simultaneous SANS/FTIR–ATR measurements

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Abbreviations: SANS, small-angle neutron scattering; FTIR, Fourier-transform infrared; ATR, attenuated total reflection; SDD, sample-to-detector distance

ABSTRACT

Due to the complicated hierarchical structure of starch, it is customary to evaluate starch retrogradation by combining several methods covering various spatial scales. However, structural analyses are typically performed individually, making correlating the structural changes at different spatial scales challenging. Therefore, this study applied a simultaneous measurement system of small-angle neutron scattering (SANS)/Fourier-transform infrared (FTIR)–attenuated total reflection (ATR) to record multiple structural changes in potato starch during retrogradation. The SANS patterns show that the shoulder-like peak becomes more pronounced with time. The peak intensity, I_{max}, representing the amount of ordered semicrystalline structures, increased over time, indicating that starch reassembled orderly on the nanoscale upon retrogradation. In the FTIR–ATR spectra, the ratio of absorptions ($R_{1042/1016}$) at 1042 and 1016 cm^{-1}, indicating the short-range ordered structure in starch, increased during retrogradation. This result indicates that the double-helix structures were reformed during retrogradation. The rate constant of the kinetic change for $R_{1042/1016}$ was larger than for I_{max}, indicating that changes in the short-range ordered structure of starch converged before the changes in the semicrystalline structure. These results indicate that the formation of double-helix structures of the amylopectin side chain and the structural change of its ordered arrangement could occur in stages during retrogradation.

Keywords: starch, retrogradation, small-angle neutron scattering, FTIR–ATR, simultaneous measurement

INTRODUCTION

Starch retrogradation is where gelatinized starch recrystallizes over time [1–3]. It is

an undesirable structural change in food processing because it significantly affects the quality of starchy foods' texture and shelf life. However, due to the complicated structure of starch, retrogradation is yet to be completely elucidated. Starch has a hierarchical structure at various spatial scales [4–6]. Starch granules are semicrystalline and have a layered structure with alternating amorphous and semicrystalline growth rings [7]. Semicrystalline growth rings have a structural periodicity comprising crystalline and amorphous lamellae, and a pair is called a cluster [8]. The crystalline regions are described by a short-range ordered structure, with a molecular order related to the double-helix structure in starch studies [9,10], and a long-range ordered structure, showing the overall order of starch crystals regarding the packing of the double-helix of amylopectin side chains [11,12]. Due to the complex hierarchical structure of starch, it is crucial to cover various spatial scales to investigate starch retrogradation.

In starch retrogradation studies, it is necessary to investigate the structural changes at each scale using appropriate analytical methods. Then, clarifying the chronological sequence of structural changes revealed in each analysis is essential for adequately understanding the structural changes in starch retrogradation. However, each experiment is typically performed individually. While gelatinization is easily controlled reproducibly by temperature and moisture content, starch retrogradation is not easily controlled. It changes over time, and it is challenging to completely replicate the results even using the same sample because the conditions and environment significantly affect the rate and degree of starch retrogradation [13]. Furthermore, it is challenging to correlate the structural information with various methods during retrogradation in hour units. To overcome this problem, freeze-dried starch samples where retrogradation is stopped are used for evaluation, but this can only provide fragmentary information on retrogradation. Therefore, monitoring starch retrogradation in real-time and natively through simultaneous measurements is valuable.

Researchers have used various simultaneous measurement systems in starch studies; for example, small-angle neutron scattering (SANS) is applied because neutrons have high penetration and low energy, avoiding damage to the sample. Doutch et al. measured the loss in lamellar order and starch gel formation using a rapid visco analyzer and SANS simultaneously [14]. Pullen et al. investigated structural and thermal changes in starch using simultaneous SANS and differential scanning calorimetry measurements [15]. Balacescu et al. developed a simultaneous SANS and Fourier-transform infrared (FTIR) method by transmission [16]. Although they attempted to apply this method to observe the starch structure, the infrared (IR) light could not penetrate the starch gel, and the measurement was insufficient. FTIR–attenuated total reflection (ATR) is also frequently used to measure structural changes in starch retrogradation [17–19]. Therefore, the ATR mode of FTIR effectively records the structural change of starch in this study.

FTIR spectroscopy is suitable for investigating the short-range ordered structure of starch, and small-angle scattering (SAS) is appropriate for examining the clusters of lamellar structures in starch. FTIR can evaluate the amount of the short-range ordered structures in starch using the absorption ratio of crystalline and amorphous regions in starch [5,20,21]. FTIR observes the environment around the atoms, and the formation of short-range ordered structures causes changes in the state of functional groups attributed to these two regions. The changes in starch crystallinity on the atomic scale due to retrogradation determined by FTIR are interpreted as changes in these two states. However, the SAS pattern of starch shows several diffraction peaks related to the periodic arrangement of clusters in the lamellar structure, and the cluster thickness or lamellar spacing can be observed [8]. The changes in the semicrystalline structure, such as clusters in starch on the nanoscale, due to retrogradation determined by SAS are interpreted as changes in the double-helix arrangement in that structure. Note that, given

104 the hierarchical nature of the starch structure, characterized by the structure on a wide
105 spatial scale, the structural information obtained by SAS differs from that obtained by
106 FTIR. Although many researchers have reported changes in short-range ordered and
107 semicrystalline structures of starch during retrogradation [22–24], the kinetic
108 relationship between them during retrogradation is unclear. Verifying these by
109 simultaneous SANS and FTIR–ATR measurements would be possible to clarify the
110 chronological sequence of structural changes in short-range order and semicrystalline
111 structures due to retrogradation.

112 This study performed simultaneous measurements to compare the kinetic
113 information of the semicrystalline structure in starch retrogradation obtained by SANS
114 with that of short-range ordered structures obtained by FTIR–ATR. This study revealed
115 the order of structural changes at different scales during starch retrogradation. It should
116 be emphasized that the simultaneous measurement method was effective in
117 quantitatively discussing the correlation between these structural changes associated
118 with retrogradation.

119 **MATERIALS AND METHODS**

120 ***Materials.*** Rice starch prepared from polished rice, *Hitomebore* (Miyagi, Japan, 2021),
121 using the cold alkaline immersion method [25], was used as the A-type starch. Potato
122 starch (Nacalai Tesque, Japan) was the B-type starch, and sweet potato starch (Wako
123 Pure Chemical, Japan) was the C-type starch.

124 ***Gelatinization of starch.*** Although D_2O is typically used as a solvent in SANS to avoid
125 the strong incoherent scattering of H_2O, the starch retrogradation rate is faster when
126 gelatinized with D_2O [26]. Thus, in this study, the samples were prepared with H_2O to
127 evaluate the starch retrogradation under natural conditions. Distilled water (3.25 mL) at
128 25 °C was added to 2.5 g of the starch sample and mixed well. The sample was
129 gelatinized immediately for 60 min at 105 °C in an autoclave (HVE-50LB,

HIRAYAMA Manufacturing Corp., Japan). The gelatinized sample was cooled at 25 °C for 30 min and was used in the following experiments.

SANS. SANS experiments were performed using the SANS-J instrument at the Japan Research Reactor 3 (JRR-3) at the Japan Atomic Energy Agency (JAEA), Japan [27]. To find the specific structural changes of starch during retrogradation by observing a wider spatial range, SANS was conducted at three sample-to-detector distances (SDDs): 2, 4, and 10 m and neutrons λ of 0.5 and 1 nm ($\Delta\lambda/\lambda$ = 10%). The gelatinized starch samples were placed in 1-mm path-length sample cells with demountable quartz windows and measured after 1 h. The SANS measurements were performed with an SDD at 2 m in the order of potato, rice, and sweet potato starches. Measurements were performed on the same sample order with SDDs of 4 and 10 m without sample exchange and repeated four times over 12 h to evaluate the structural changes in starch. Each raw scattering dataset was corrected for detector sensitivity, electronic background, and empty cell contribution and converted to scattering cross-section data using the software Igor Pro 9.

Simultaneous SANS/FTIR–ATR measurements. Simultaneous SANS/FTIR–ATR measurements were performed using the SANS-J instrument at the JAEA, Japan, as described in Section of *SANS* [28]. SANS measurements were performed using an SDD of 4 m and neutrons λ of 0.5 and 1 nm ($\Delta\lambda/\lambda$ = 10%) to achieve a maximum dynamic Q-range of 6×10^{-2}–0.8 nm^{-1}. In situ FTIR investigations were conducted during the SANS measurements using an FTIR spectrometer (VIR 200, JASCO Co.) installed at a sample position in an appropriate geometry, enabling simultaneous sample irradiation using IR and neutron coaxial beams (Fig. 1). The IR beam (the white arrows in Fig. 1) from the FTIR instrument on the right side, whose direction is adjusted by a mirror, is incident on the ATR prism in the center at 45°, is reflected five times on the sample surface, and exits to the IR detector on the left side. The neutron beam (the black arrows

156 in Fig. 1) is irradiated on the sample. The scattered neutron beam passes through the
157 ATR prism to the neutron detector. The ATR sample holder is made of copper and is
158 divided into a lid and a body part. An O-ring between them seals the sample space. A
159 trapezoidal ZnSe prism (base angle: 45°, length: 30 mm, width: 10 mm, thickness: 3
160 mm, refractive index: 2.4) for ATR measurements is attached to the lid, and a circular
161 ZnSe window (diameter: 22 mm, thickness: 2 mm) to the body. The sample thickness is
162 adjusted to 1 mm. One hour after completing the cooling, the gelatinized potato starch
163 sample was placed on the ATR crystal surface. The SANS measurement was repeated
164 12 times continuously, alternating 30 min sample and 3 min transmittance
165 measurements (Fig. 1). Section of *SANS* describes the SANS data analysis. The FTIR
166 measurement was repeated 37 times every 15 min continuously, with a scan range of
167 400–4000 cm^{-1} at a resolution of 4 cm^{-1}. We determined the peak positions by the
168 second derivative of the obtained spectra using the spectra manager analysis software
169 provided by JASCO (3rd polynomial order and 15 smoothing points of
170 Savitzky-Golay).

171 **RESULTS AND DISCUSSION**

172 ***Changes in the SANS patterns for all starches.***

173 The starches are classified into A-type, B-type, and C-type based on X-ray diffraction
174 (XRD) patterns, each differing in retrogradation rate [25]. This study measured rice
175 starch showing A-type, potato starch showing B-type, and sweet potato starch showing
176 C-type for SANS to select starch samples that change hourly. Fig. 2 shows the SANS
177 patterns (I, Q) of the starch samples, where I and Q represent the scattering intensity and
178 amplitude of the scattering vector, respectively. No change in the rice starch scattering
179 pattern with time was observed in all Q ranges in an hour. However, the potato and
180 sweet potato starch scattering intensities increased with time in the Q ranges from 0.08
181 to 0.6 nm^{-1} and 0.03 to 0.5 nm^{-1}, respectively. The retrogradation rate is the fastest for

B-type starch and the slowest for A-type starch [25]. This trend correlates with a previous XRD study [25]. The sweet potato starch scattering intensity increased in the lower Q region compared with that of potato starch, and the changes in the SANS patterns differed. The intensity gradually decreases from the low Q region to high Q region, reflecting changes in the aggregated structures in the starch samples [29]. SANS intensity is correlated with the density of nuclei, and the strong SANS intensity reflects enhanced ordered aggregated structures. Thus, the increase in intensity in the lower Q region indicates that the aggregates formed by retrogradation grew for sweet potato starch. A shoulder-like peak appeared at Q = 0.2–0.3 nm^{-1} in potato starch, consistent with that of starch recrystallization due to retrogradation reported by small-angle X-ray scattering (SAXS) [23,30]. Although the restored structure differs from the native one, this result indicates that semicrystalline-like structures are formed. Simultaneous measurements using potato starch were conducted to investigate the relationship between lamellae and short-range order during retrogradation. Simultaneous SANS/FTIR–ATR measurements were performed in the subsequent section using an SDD of 4 m for potato starch to focus on the shoulder peak of starch because the change can be observed in the Q region.

Changes in SANS patterns during simultaneous measurements.

Fig. 3A shows potato starch SANS patterns (I, Q) for simultaneous SANS and FTIR–ATR measurements. As in Section of *Changes in the SANS patterns for all starches*, the scattering intensity gradually increases, and a shoulder-like peak appears at Q = 0.2–0.3 nm^{-1}. The shoulder-like peak becomes more pronounced with time, indicating that the semicrystalline-like structure appears during retrogradation, the same as Fig. 2B-2.

Fig. 3B illustrates the transmittance transition over time. The transmittance values, which remained unchanged at approximately 36%, confirm the hermeticity of the

sample cell. The very low transmittance values might result from the incoherent scattering of H_2O. As mentioned in Section of *Gelatinization of starch*, in this study, the samples were prepared with H_2O to evaluate the starch retrogradation under natural conditions. The incoherent scattering of H_2O has a constant intensity irrespective of Q, meaning that the Q dependence of the obtained SANS profile can be attributed to coherent scattering from the starch structure and can be used for structural analysis.

The shoulder-like peak based on the semicrystalline model was quantitatively analyzed by model fitting the SANS profile based on the Cauchy and Power law functions (Eq. 1) [8,24],

$$I(Q) = I_{max}\left[1 + 4\left(\frac{Q-Q_1}{\Delta Q}\right)^2\right]^{-1} + AQ^{-\alpha} + B \quad (1)$$

where I_{max} is the peak intensity, Q_1 is the maximum peak position, ΔQ is the full width at half maximum of the peak, α is the fractal coefficient, and A and B are positive adjustable parameters.

Many SAXS studies have focused on I_{max}, which relates to the amount of semicrystalline structures, Q_1, reflecting the lamellae spacing, and α, representing the fractal dimension [24,31,32]. When the data were initially fitted without the parameter fixed, ΔQ, A, and B were near constant regarding time. This study improved the accuracy of the I_{max}, Q_1, and α values by fitting the SANS patterns with ΔQ, A, and B fixed with the averaged values of all fitting data (ΔQ = 0.317802 nm^{-1}, A = 0.62911, and B = 2.722267) (Fig. 3A). Furthermore, Q_1 was obtained by fitting the SANS patterns with I_{max} and α fixed to improve accuracy. Fig. 4 shows the changes in the I_{max}, Q_1, and α values obtained by Eq. (1) over time. The I_{max} value was initially 1.42 but increased over time and reached 6.01. I_{max} depends on the amount of ordered semicrystalline structures and enables a qualitative comparison of the degree of order of starch molecules [8,32]. This result shows that the amount of regularly arranged double helical structures increased over time, indicating that the starch was regularly

reassembled at the nanoscale during retrogradation. The Q_1 value decreased slightly from 0.273 at the beginning to approximately 0.206 at the end, indicating that the peak position shifted from the high to low Q regions. This result indicates that the spacing between lamellae gradually increases slightly during retrogradation. In previous studies, the α value was used as an indicator to evaluate the compactness of the starch structure [23,24]. The α value increased slightly from 1.44 to approximately 1.52, which is inferred to be the increased compactness of the starch structure by retrogradation. These results correlate with the trends observed in previous SAXS studies [24].

FTIR–ATR spectral changes during simultaneous measurements.

Fig. 5A shows the FTIR spectral changes of potato starch during retrogradation. The FTIR spectrum shape was similar to that of the starch hydration samples in a previous study [19]. Absorbance at 1047 cm^{-1} and 1022 cm^{-1} reflects the crystalline and amorphous regions of starch, respectively [9]. These bands indicate the bending of the –COH and $–CH_2$ of starch. The intensity ratio of the 1047 peak to the 1022 one, $R_{1047/1022}$, is frequently used to evaluate the crystallinity and the short-range order in starch samples [5,20,21]; in this study, the peak positions were determined at 1042 and 1016 cm^{-1} by the second derivative (Fig. 5B). The $R_{1042/1016}$ value increased over time (Fig. 5C), confirming that the short-range ordered structure in starch increases during retrogradation, consistent with previous studies [22,33–35]. Bai et al. reported that the decrease in the $R_{1047/1022}$ value indicates a reduction in the formation of double-helix structures [35]. The changes in $R_{1042/1016}$ indicate that the formation of double-helix structures in starch during retrogradation, which led to a change in the frequency of the bending of the –COH and $–CH_2$ of starch.

The broad absorption from 3000 to 3700 cm^{-1} derives from the O–H stretch modes of starch and water [22,36–38]. As in a previous study, the water adsorption band in the

hydrated starch were overlapped with the peaks related to the starch from 3000 to 3700 cm^{-1} [19]. Although it can be seen that the absorbance increased over time in this frequency range (Fig. 5A), it is difficult to distinguish whether this spectral change is due to starch and/or water. On the other hand, the absorption peak at approximately 1640 cm^{-1} is related to the water content of the sample and represents the OH-bending vibration of water molecules without overlapping with absorbance of the starch [36,39]. This absorbance increased over time as well (Fig. 5A). The peak position was determined at 1642 cm^{-1} by the second derivative (Fig. 5D). And then, the second derivatives of absorbance at 1642 cm^{-1} (D_{1642}) were calculated to eliminate the effect of the baseline, and the D_{1642} value decreased over time (Fig. 5C). Because the ATR method observes the surface of the sample, the decrease in D_{1642} should mean an increase in the observed water absorbance. In addition, the no change in the SANS transmittance confirms the hermeticity of the sample cell (Fig. 3B); therefore, there is no influence from water evaporation during the measurement, and then the change in D_{1642} suggests that water in the starch is leaving the sample and collecting on the surface of ATR prism. Furthermore, potato starch recrystallizes in hour units [25]. The recovery of the crystalline structure means that the hydrogen bonds between the starch and water are broken, and this implies the release of water. For these reasons, water release can occur within the measurement time of this study, and the change in D_{1642} is considered to represent water release due to recrystallization. It was also observed that retrogradation expels the water from the gelatinized starch [40]. Zhang et al. reported that in LF-NMR imaging, the water in starch gel migrated outward from the center of the gel with storage time [41]. These are consistent with our results. Fig. 5E shows that absorbance at 1042 cm^{-1} and 1016 cm^{-1} was reduced, which could be due to the aggregation of starch and the release of water by retrogradation, reducing the starch density on the sample surface. However, this phenomenon contributes less to these

ratios and does not influence the quantification of short-range ordered structures as determined by FTIR–ATR. The FTIR–ATR results showed an increase in the short-range ordered structures and water release of the sample by retrogradation. Simultaneous SANS/FTIR–ATR measurements with a tightly sealed sample cell allow us to track the structural changes in starch and changes in water release over time simultaneously.

Relationship between structural changes at different spatial scales and dehydration. The rate constants of structural changes for each parameter can be determined using I_{max} from SANS and $R_{1042/1016}$ and D_{1642} from FTIR–ATR. Analyzing them could provide information on the order in which each structural change occurs in starch retrogradation. Fig. 6 plots the I_{max} determined by SANS and $R_{1042/1016}$ and D_{1642} determined by FTIR–ATR. Many researchers have used the Avrami equation to evaluate the kinetic changes in the ordered and crystalline structures of retrograded starch [42,43]. The retrogradation degree of potato starch converges to equilibrium [25]. Therefore, we easily compared these rate constants by fitting the data using a modified version of the Avrami equation (Eq. 2), where the exponent was considered equal to 1, as in previous studies [44,45], and calculated the time to obtain half the equilibrium value,

$$\frac{a-y}{a-y_i} = \exp(-kt) \quad (2)$$

where a is the equilibrium value, y is I_{max} for SANS and $R_{1042/1016}$ and D_{1642} for FTIR–ATR, y_i is the initial of y, k is the rate constant, and t is the storage time.

The rate constant k values were 0.0018 ± 0.0006 min^{-1} for I_{max}, 0.0050 ± 0.0006 min^{-1} for $R_{1042/1016}$, and 0.0064 ± 0.0010 min^{-1} for D_{1642}. The time to obtain half the equilibrium value was 383.3 min for I_{max}, 138.0 min for $R_{1042/1016}$, and 108.3 min for D_{1642}. The double-helix formation, regular helix alignment, and water release proceed simultaneously after gelatinization but at different rates. The time required to obtain half

312 the equilibrium value for $R_{1042/1016}$ was less than for I_{max}, indicating that the changes in
313 the short-range ordered structure in starch observed by FTIR–ATR converge before
314 changes in the nanostructure observed by SANS. These results show that the formation
315 of the double-helix structures in the amylopectin side chain and the structural change of
316 its ordered arrangement could occur in stages. In other words, there is a transient state
317 during retrogradation where double helixes are formed but with disordered
318 arrangements. Half the equilibrium of D_{1642} is faster than the other two due to the
319 structural changes in starch. Water release from the gelatinized starch was completed
320 before the structural changes in the starch during retrogradation.
321

CONCLUSIONS

This study evaluated multiple structural changes during potato starch retrogradation using a simultaneous SANS/FTIR–ATR measurement system. The SANS shoulder-like peak becomes more pronounced over time, indicating that retrogradation forms semicrystalline-like structures. The SANS analysis results based on the Cauchy and Power law functions showed that I_{max} increased over time, revealing that starch reassembled orderly on the nanoscale upon retrogradation, increasing the amount of orderly arranged double helical structures. In the FTIR–ATR spectra, $R_{1042/1016}$ increased and D_{1642} decreased over time, confirming an increase in the short-range ordered structures in starch and water release of the sample during retrogradation. Comparing the time required to obtain half the equilibrium value showed that water release was completed before the structural changes in the starch during retrogradation. Furthermore, changes in the short-range ordered structures of starch observed by FTIR–ATR converged before changes in the semicrystalline structure observed by SANS. These simultaneous SANS/FTIR–ATR measurement results show that the formation of the double-helix structures of the amylopectin side chain and the structural change of its ordered arrangement could occur in stages during retrogradation. It should be emphasized that the simultaneous measurement method was effective in quantitatively discussing the correlation between these different structural changes associated with retrogradation, which is a remarkable result in the technical aspect of this study.

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DECLARATION OF COMPETING INTEREST

The authors declare no conflict of interests.

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