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Inclusion Compound Formation of Amylose by Sealed-Heating with Salicylic Acid Analogues

TOMONOBU UCHINO*, YUICHI TOZUKA, TOSHIO OGUCHI and KEIJI YAMAMOTO
Graduate School of Pharmaceutical Sciences, Chiba University, 1-33 Yayoi-cho, Inage-ku, Chiba 263-8522, Japan

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Abstract

The inclusion compounds of linear amylose and salicylic acid analogues prepared by sealed-heating were evaluated by using powder X-ray diffraction and infrared spectroscopy. Sealed-heating of amylose and either salicylamide or benzoic acid induced the amylose structural change from the 6_1- to the 7_1-helix structure, while sealed-heating with *o*-toluic acid, *o*-chlorobenzoic acid and *o*-nitrobenzoic acid induced the change from the 6_1- to the 8_1-helix structure. In contrast to the results in the salicylic acid system, the amylose helix type of sealed-heated sample was not varied by the load of the guest compound, but was fixed only by the guest species. No change of the helical structure of amylose depending on the amylose molecular weight was observed. From the comparison of inclusion compound formation among different guest systems, it was found that the higher vapor pressure at 100 °C of the guest resulted in faster inclusion compound formation. The vapor pressure of the guest compound would be an important factor affecting the progress of inclusion compound formation.

Introduction

Starch is composed of two main components: amylose, a mainly linear polymer of α-1,4 linked D-glucospyranoses, and amylopectin, a highly branched polymer of α-1,4 and α-1,6 linked glucospyranoses. Amylose derived from starch, however, contains α-1,6 linked branching in its structure and has a wide distribution of molecular weight. At present, linear amylose of controlled molecular weight can be prepared by enzymatic synthesis and has become available [1–3].

It is well known that amylose can form inclusion compounds with guest molecules such as iodine, alcohols and lipids whereas amylopectin forms these complexes only weakly or not at all [4–5]. When amylose molecules accommodated the guest, amylose took a single helix structure called V-amylose. Preparation of inclusion compounds of amylose is usually conducted by mixing an aqueous solution of amylose with an alcoholic solution of the guest compound [6–13]. The complexation of the amylose fraction with guests is also suitable for the separation of amylose from amylopectin in starch.

Previously, we applied the sealed-heating method to amylose-guest systems by using salicylic acid as a guest [2]. Two helix types of inclusion compound, 7_1- and 8_1-helices of amylose, were obtained by sealed-heating depending on the load of salicylic acid. We also reported that the heating temperature, heating time and water content in the physical mixture affected the inclusion compound formation [3].

The aims of the present study were to investigate the possibility of the inclusion compound formation between amylose and salicylic acid analogues, and to examine the effect of guest property and the molecular weight of amylose on the helical structure change of amylose by the sealed-heating. Figure 1 shows the structure of the guest molecules used in this experiment. These guests were selected because these molecules are similar to salicylic acid in terms of molecular size and shape and form intermolecular hydrogen bonds between carboxyl groups in the crystal [14–20].

Experimental

Materials

Enzymatically synthesized amyloses were received as a gift from Nakano Vinegar Co., Ltd. and Ezaki Glico Co., Ltd. The weight average molecular weight of the amyloses were 70,000 (AS70), 100,000 (AS100) and 150,000 (AS150). Salicylic acid (SA), salicylamide (SAM), benzoic acid (BA), *o*-aminobenzoic acid (*o*-ABA), *o*-chlorobenzoic acid (*o*-CBA), *o*-toluic acid (*o*-TA) *o*-nitrobenzoic acid (*o*-NBA) and *o*-anisic acid (*o*-AA) were used as guest compounds. All guest compounds were of analytical reagent grade and were purchased from Nacalai Tesque, Inc. (Kyoto Japan). The particle size of AS150 and guest molecules was controlled between 42.5 and 150 μm by sieving.

Sample preparation and sealed-heating procedure

All amyloses were stored at 25 °C and 33% RH to control the water content of amylose. Physical mixtures were prepared at various mixing ratios of AS150 and guest in a glass vial

* Author for correspondence.

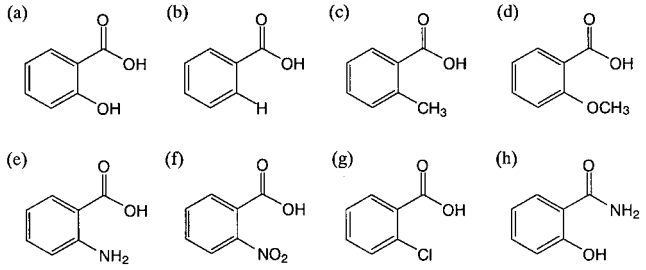

Figure 1. Chemical structure of guest molecules. (a) salicylic acid (SA), (b) benzoic acid (BA), (c) *o*-toluic acid (*o*-TA), (d) *o*-anisic acid (*o*-AA), (e) *o*-aminobenzoic acid (*o*-ABA), (f) *o*-nitrobenzoic acid (*o*-NBA), (g) *o*-chlorobenzoic acid (*o*-CBA), (h) salicylamide (SAM).

using a mixer. The water content of the physical mixture was determined by the Karl Fischer method using Aquacounter :AQ-5 (Hiranuma, Ibaraki, Japan). A physical mixture (ca. 250 mg) was put into a 2 mL glass ampoule and heated at various temperatures for a definite time after sealing of the glass ampoule. The mixing ratio of AS150 and guest was fixed as the ratio of 1∼4 guest molecules to one helical unit of 7_1- or 8_1-helix amylose. The temperature of the glass ampoule in the oven was monitored with thermocouples.

Powder X-ray diffraction

The powder X-ray diffractograms were measured on a Rigaku Miniflex diffractometer (Cu-Kα, voltage 30 kV, current 15 mA, scanning speed 4 degrees min^{-1}).

Infrared (IR) spectroscopy

Infrared measurements were carried out with a model 230 FT-IR spectrometer (Jasco, Tokyo, Japan) using the KBr method.

Determination of included amount of guest compound

To remove the excess of the free guest, sealed-heated samples were washed with diethylether. Treated samples were dispersed into phosphate buffer (pH = 6.8) and sonicated for 30 min. After filtration through a membrane filter (1.0 μm pore size), the guest concentration in each solution was determined spectrophotometrically at 296.0, 300.4, 223.8, 202.8, 212.8, 201.6 and 266.8 nm for SA, SAM, BA, *o*-ABA, *o*-CBA, *o*-TA and *o*-NBA, respectively.

Results and discussion

Effect of guest species on inclusion compound formation of amylose by sealed-heating

We have already reported that the inclusion compound of either the 7_1- or 8_1-helical structure was formed between AS1310 (m.w.= 1,310,000) and SA during the sealed-heating process [2]. With regard to the AS150-SA system, complexation of the 7_1- and 8_1-helix structure depended on the load of SA induced by sealed-heating.

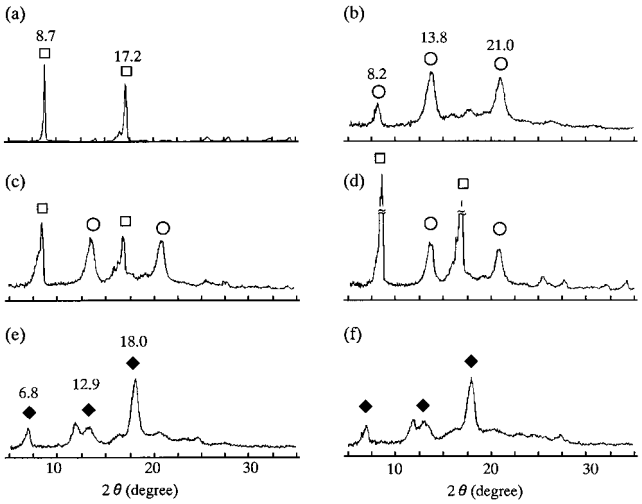

Figure 2. Change in the powder XRD patterns of AS150 : SAM systems during the sealed-heating process. (a) SAM crystal, (b) AS150, (c) Physical Mixture (PM) of 7-Glucose Units (GU) : SAM = 1 : 1, (d) PM of 8-GU : SAM = 1 : 2, (e) sealed-heated sample of (c) at 120 °C for 6 h, (f) sealed-heated sample of (d) at 120 °C for 1 h.

In order to examine the effect of the guest species on the inclusion compound formation of amylose by sealed-heating, SA analogues were used as guest compounds. SAM, BA, *o*-ABA, *o*-CBA, *o*-TA, *o*-NBA and *o*-AA were selected as SA analogues as shown in Figure 1. Figure 2 shows the powder XRD patterns of SAM, AS150, two kinds of physical mixtures between AS150 and SAM (7 glucose units (GU) : SAM = 1 : 1 and 8 GU : SAM = 1 : 2) and their sealed-heated samples. Salicylamide crystals showed crystalline diffraction peaks at 8.7 and 17.2°. AS150 showed crystalline diffraction peaks at 2θ = 8.2, 13.8, and 21.0° due to the 6_1-helix structure of amylose (as indicated by the open circles). The diffraction patterns of the physical mixtures were simply a superposition of the patterns of AS150 and SAM crystals. After the sealed-heating at 120 °C for 6 h in the 1 : 1 (7_1-helical turn : SAM) physical mixture, new diffraction peaks at 6.8, 12.9, and 18.0° (as indicated by closed diamonds) were observed, whereas the diffraction peaks due to SAM and 6_1-helical structure of amylose had disappeared. The diffraction patterns of the sealed-heated samples coincided with the 7_1-helix structure of amylose as reported in the previous paper, respectively [2]. The 8_1-helix structure of amylose was not observed in the 8 GU : SAM= 1 : 2 system. These results indicated that the sealed-heating led to a structural change of amylose from 6_1-helix to 7_1-helix by including SAM. The same results were obtained by using BA and *o*-ABA as guest compounds (data not shown).

On the other hand, different results were obtained by using *o*-CBA, *o*-TA and *o*-NBA. The change in the powder XRD patterns of the AS150 and *o*-NBA system is shown in Figure 3. New characteristic diffraction peaks at 6.2, 12.6, and 16.3° (as indicated by closed stars) appeared in the diffractgram after the sealed-heating at 120 °C for 1 h in the 8 GU : *o*-NBA = 1 : 2 system. It was recognized that the helical structure of amylose was changed from 6_1- to 8_1-helix, since new diffraction peaks were consistent with the 8_1-helix structure of amylose as reported in the previous paper [2]. X-

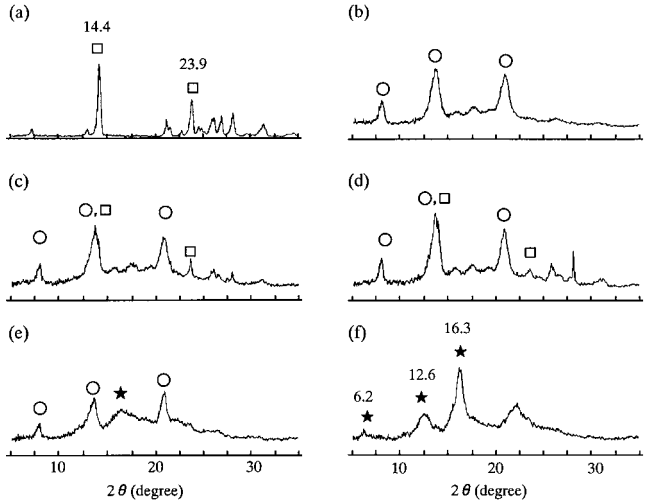

Figure 3. Change in the powder XRD patterns of AS150 : *o*-NBA systems during the sealed-heating process. (a) *o*-NBA crystal, (b) AS150, (c) PM of 7-GU : *o*-NBA = 1 : 1, (d) PM of 8-GU : *o*-NBA = 1 : 2, (e) sealed-heated sample of (c) at 120 °C for 6 h, (f) sealed-heated sample of (d) at 120 °C for 1 h.

ray diffraction peaks due to unreacted 6_1-helix as well as the 8_1-helix structure were observed in 7 GU : *o*-NBA = 1 : 1 systems. These results suggested that the 7_1-helix inclusion compound was not formed in the sealed-heated sample of the 1 : 1 system.

To investigate the molecular state of the guest in the sealed-heated samples, IR measurements were carried out. Figure 4 illustrates the change in IR spectra of the AS150 : *o*-NBA (8 GU : *o*-NBA = 1 : 2) system. The physical mixture showed the bands at 1679 cm^{-1} for carbonyl symmetric stretching vibration and at 1531 cm^{-1} for nitro asymmertic stretching vibration of *o*-NBA. In the sealed-heated sample, the carbonyl symmetric and nitro asymmetric stretching bands were shifted to a higher wave number at 1728 and 1537 cm^{-1}, respectively (Figure 4d). The same carbonyl stretching band shifts were observed in the other SA analogue systems (data not shown). It was reported that the guest molecules used in this study have an intermolecular hydrogen bond between carboxyl groups in the crystal [14–20]. Therefore, alteration of IR bands indicated that the molecular state of SA analogues changed from the crystalline state to the included state in the helical structure of amylose by breakage of the hydrogen bond between carboxyl groups.

Comparison of inclusion behavior of the o-NBA system with that of the SA system

To compare the inclusion behavior for the SA and *o*-NBA systems, physical mixtures of AS150 and either SA or *o*-NBA(AS150 : guest = 1 : 2) were heated in a glass ampoule at 100 °C for various times. Changes in the powder XRD patterns are shown in Figure 5. In the case of the SA system, the intensity of the diffraction peak at $2\theta = 21.0°$ due to the 6_1-helical structure decreased quickly, and the intensity of the diffraction peak at 18.0° due to the 7_1-helical structure increased at the sealed-heating time of 1 and 3 min in the

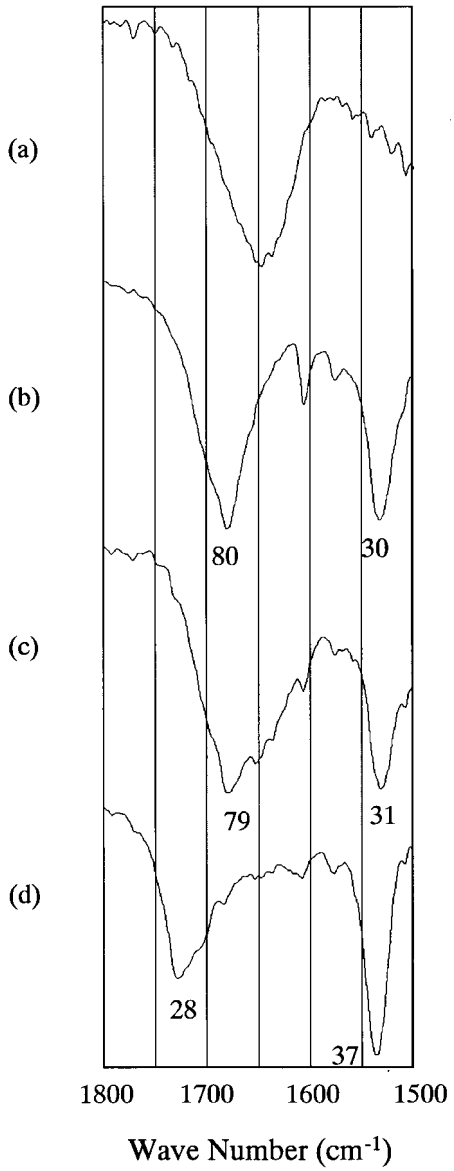

Figure 4. Change in the IR Spectra of AS150 : *o*-NBA systems during the sealed-heating process (Sealed-heating was carried out at 100 °C for 3 h.). (a) AS150 intact (b) *o*-NBA crystal, (c) PM of 8-GU : *o*-NBA = 1 : 2, (d) sealed-heated sample of (c) at 100 °C for 3h.

AS150 : SA system (Figure 5a). After sealed-heating for 10 and 30 min, the intensity of the diffraction peak at 18.0° due to the 7_1-helical structure decreased with the increase in the sealed-heating time. Finally a peak at 16.3° due to the 8_1-helical structure was observed. This inclusion behavior indicated that the helical structure of AS150 changed from 6_1- to 7_1-helix at first and then to 8_1-helix in the AS150 : SA system.

In the AS150 : *o*-NBA system (Figure 5b), the diffraction peaks due to 6_1-helix disappeared with increasing the heating time. After 30 min heating, the diffraction peaks due to 8_1-helix appeared, indicating the formation of the inclusion compound between AS150 and *o*-NBA. These results indicate that AS150 directly changed from a 6_1- to an 8_1-helical structure without forming a 7_1-helix structure. In the case of the mixture of AS150 : *o*-TA and *o*-CBA, the helical

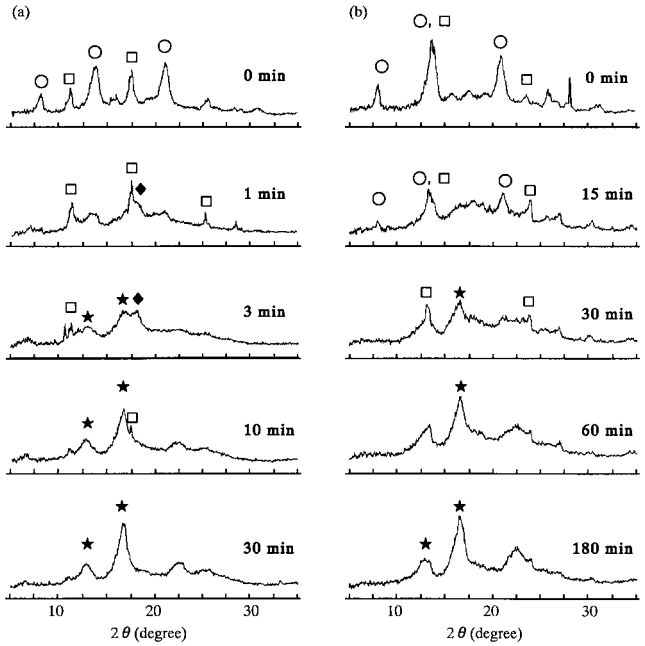

Figure 5. Change of powder XRD patterns of AS150: (a) SA and (b) *o*-NBA systems by sealed-heating at 100 °C as a function of heating time: heating time (a) from top to bottom, 0 min (PM), 1, 3, 10 and 30 min, (b) from top to bottom, 0 min (PM), 15, 30, 60 and 180 min.

structure of AS150 directly changed from 6_1- to 8_1-helix as well. From the results of Figure 5, it was found that the inclusion behavior of SA to amylose was different from the cases of the other guests.

Determination of the binding ratio of the guest to amylose

To remove the unreacted guest molecules, each sealed-heated sample was washed with diethylether and dried in a desiccator. The washed samples were dispersed in phosphate buffer (pH = 6.8) and the amounts of the included guest were determined spectrophotometrically. The results are summarized in Table 1. As reported for the SA system using AS1310 in the previous paper [2], the 7_1- and 8_1-helix structure of AS150 included one or two SA molecules, respectively. It was recognized that two *o*-NBA molecules were included per 8_1-helical turn in the *o*-NBA system. Interestingly, the 7_1-helix structure included one guest molecule and the 8_1-helix structure included two guest molecules. In the BA, SAM and *o*-ABA systems, one guest molecule was included in one 7_1-helical turn. In the *o*-TA, and *o*-CBA systems, two guest molecules were included per 8_1-helical turn.

It was reported that channel type structures of β- and γ-cyclodextrin have analogous crystal structures to 7_1- and 8_1-helix amylose [9], and the inside diameters of the 7_1- and 8_1-helix amylose can be assumed to be 7.0 and 8.5 Å, respectively [21]. Table 1 also represents the relationship between the helix type of inclusion compound and the diameter of guest molecules. With regard to the minimum cross sectional diameter of guest molecules, the small size guests (<7.0 Å) formed a 7_1-helix inclusion compound, and large size guests (>7.0 Å) formed the 8_1-helix inclusion compound. It could be reasonable that the diameter of *o*-AA

Table 1. Relationship between helix type of inclusion compounds and characteristics of guest

Guest	Helix type of inclusion compound	Binding ratio of guest to 7_1- or 8_1-helix	Minimum cross sectional diameter of guest molecule (Å)
SA	7_1-Helix	1.1	6.5
	8_1-Helix	2.0	
BA	7_1-Helix	1.0	5.7
SAM	7_1-Helix	1.0	6.7
o-ABA	7_1-Helix	1.1	6.8
o-TA	8_1-Helix	2.0	7.2
o-CBA	8_1-Helix	1.8	7.7
o-NBA	8_1-Helix	2.0	8.2
o-AA	*	–	8.5

*Inclusion compound was not found.

was too great to form an 8_1-helix inclusion compound. This indicates that the molecular size of the guest affects significantly the determination of the helix type of the amylose inclusion compound. In the SA system, on the other hand, amylose could form either a 7_1- or an 8_1-helix inclusion compound depending on the amount of SA load.

Effect of the molecular weight of amylose on inclusion compound formation

Godet *et al.* reported that amylose was able to form inclusion compounds with palmitic acid depending on the degree of polymerization (DP) of amylose by the selective precipitation method. When amylose of DP 900 was used, an inclusion compound was formed with palmitic acid, while inclusion compound formation failed for amylose of DP 20 [12].

To investigate the effect of the molecular weight of amylose on inclusion compound formation, physical mixtures between three kinds of amylose and various guests were heated at 100 °C for 3 h in glass ampoules. The relationship between the molecular weight of amylose and the helix type obtained after sealed-heating with various guests is summarized in Table 2. All amyloses formed crystalline inclusion compounds by sealed-heating with all guests used. No difference of helical structure of amylose depending on the molecular weight of amylose was observed. Consequently, the effect of the molecular weight of amylose on the inclusion compound formation was not observed in these systems.

Effect of vapor pressure of the guests on inclusion compound formation

In order to compare the rate of inclusion compound formation with the different guest species, physical mixtures with amylose and either *o*-NBA, *o*-CBA or *o*-TA, which formed an 8_1-helix inclusion compound were heated in a glass ampoule at 100 °C for various times. The included amount of guests in the 8_1-helix amylose was determined after washing the sealed-heated samples with diethylether. The change in

Table 2. Relationship between molecular weight of amylose and helix type obtained after sealed-heating with various guests at 100 °C for 3 h

Amylose (molecular weight)	Helix type of amylose for various guests		
	SA	BA	*o*-NBA
AS70 (70,000)	7_1-Helix 8_1-Helix	7_1-Helix	8_1-Helix
AS100 (100,000)	7_1-Helix 8_1-Helix	7_1-Helix	8_1-Helix
AS150 (150,000)	7_1-Helix 8_1-Helix	7_1-Helix	8_1-Helix

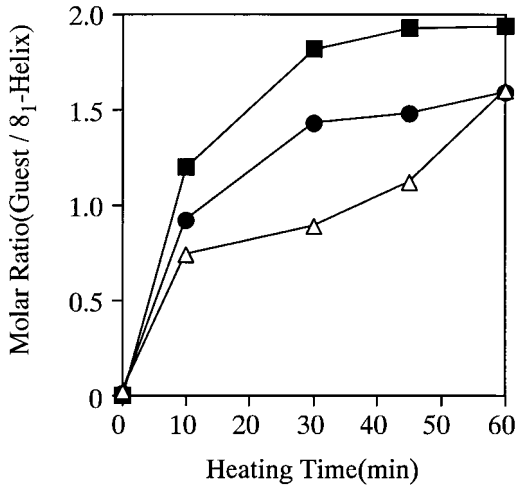

Figure 6. Change in the molar ratio of guest to an 8_1-helix as a function of heating time at 100 °C in ampoules, ■: *o*-TA, ● : *o*-CBA, △: *o*-NBA.

the binding ratio of guests to 8_1-helix amylose depending on the sealed-heating time is illustrated in Figure 6. The rate of inclusion compound formation was in the order: *o*-TA > *o*-CBA > *o*-NBA. Sidwick reported that the vapor pressure of *o*-TA, *o*-CBA and *o*-NBA at 100 °C were 0.97, 0.18 and 0.02 mmHg, respectively [22]. The rate of the inclusion compound formation showed the same order with the vapor pressure of the guests, that is, the vapor pressure of the guests could affect the rate of the 8_1-helix inclusion compound formation. The mixture of sodium benzoate, a nonsublimable guest, with AS150 was heated in a glass ampoule to investigate the effect of guest sublimation on inclusion compound formation. No significant change of powder XRD patterns and IR spectra between the physical mixture and the sealed-heated sample was observed. This indicated that sodium benzoate was not included in AS150 by sealed-heating. Nakai *et al.* reported that sublimation of the guest was an important factor in the inclusion compound formation in cyclodextrin-drug systems during the sealed-heating process [23]. These results indicated that inclusion compound formation between amylose and guests by sealed-heating was strongly affected by sublimation of the guest.

Conclusion

Although it was reported that aromatic carboxylic acids were not included in the amylose by the selective coprecipitation method [8], the sealed-heating process led to formation of the inclusion compound between AS150 and SA analogues. The change from the 6_1- to the 7_1-helix structure of AS150 was induced by sealed-heating with BA, SAM and *o*-ABA. 8_1-helix inclusion compounds were formed between AS150 and either *o*-TA, *o*-CBA or *o*-NBA. Inclusion compound formation was not observed for the AS150 and *o*-AA systems. It was found that the molecular size of the guest played an important role in the determination of amylose helix types. Only in the case where SA was used as guest compound, AS150 formed both 7_1- and 8_1-helix structures depending on the load of SA during the sealed-heating process. The rate to form inclusion compound was affected by the vapor pressure of the guest materials.

Acknowledgement

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