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# Inclusion Complex Formation between High Amylose Corn Starch and Alkylresorcinols from Rye Bran

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**ABSTRACT**

The formation of high amylose corn starch (HACS)-alkylresorcinol (AR) inclusion complexes was demonstrated using HACS and a crude AR extract from rye bran, and was confirmed by complementary characterization techniques. Inclusion complex was extracted using hot 2-propanol/water (3:1), and thirteen different AR homologs were identified in the 2-propanol/water extract. However, this extraction regime was insufficient to remove all guests from the inclusion complexes, but did result in annealing of the V-type crystallinity, yielding an endotherm with higher onset and peak temperatures and dissociation enthalpy. The remaining guest compounds were recovered by disruption of the inclusion complexes with DMSO followed by liquid-liquid extraction. A total of nine AR homologs were recovered and two saturated long alkyl chain AR homologs (C21:0 and C19:0) were obviously enriched in the complexes compared to the crude AR extract. The formation of HACS-AR inclusion complexes may have the potential to inhibit staling in baked goods.

**Keywords:** Starch; Amylose; Alkylresorcinols; Inclusion complexes; Rye bran

## 1. Introduction

Alkylresorcinols (ARs) are phenolic lipids found in the bran fractions of cereal grains, such as rye (360-3200 μg/g), triticale (580-1630 μg/g), wheat (317-1010 μg/g), and barley (44-500 μg/g), belonging to the Poaceae or Gramineae family (Kozubek & Tayman, 1999; Ross & Kochhar, 2009; Ross, Shepherd, Schüpphaus, Sinclair, Alfaro, Kamal-Eldin, et al., 2003). ARs are amphiphilic 1, 3-dihyroxybenzene species, consisting of an odd-numbered (15-25) alkyl chain substituted at position 5 of the benzene ring. In general, the alkyl chains are mostly saturated, yet, up to 20% of the total ARs in rye contain unsaturated chains (Kozubek & Tayman, 1999; Ross, Åman, & Kamal-Eldin, 2004). ARs are reported to have biological activities (e.g., anticancer, antimicrobial, and antioxidant activities), which has been comprehensively reviewed in the literature (Kozubek & Tayman, 1999; Ross, 2012b).

Starch is a mixture of two polymers: amylose (a predominantly linear polymer of glucose) and amylopectin (a branched polymer of glucose). Starch, especially the amylose component, is known to form inclusion complexes with many compounds, including alcohols (Fanta, Felker, & Selling, 2016; Lorentz, Pencreac'h, Soultani-Vigneron, Rondeau-Mouro, de Carvalho, Pontoire, et al., 2012), aroma-active compounds (Pozo-Bayon, Biais, Rampon, Cayot, & Le Bail, 2008), fatty acids (Cao, Woortman, Rudolf, & Loos, 2015), and salicylic acid (Oguchi, Yamasato, Limmatvapirat, Yonemochi, & Y., 1998). These complexes play a crucial role in the textural and structural stability of starch-based products (Biliaderis, Page, Slade, & Sirett, 1985), and have been proposed as delivery systems for guest molecules such as conjugated linoleic acid (Lalush, Bar, Zakaria, Eichler, & Shimoni, 2005) and long chain unsaturated fatty acids (Gelders, Vanderstukken, Goesaert, & Delcour, 2004; Riisom, Krog, & Eriksen, 1984). Most commonly, the interaction between amylose and monoacyl lipids is characterized by amylose chains forming

left-handed single helices that may crystallize into the so-called $V_6$-type crystalline forms (Obiro, Sinha Ray, & Emmambux, 2012). The inner surface of the amylose helix is lined with methylene groups and glycosidic linkages resulting in a hydrophobic helical cavity, while the glycosyl hydroxyl groups located on the helical exterior produce a hydrophilic surface (Cheng, Luo, Li, & Fu, 2015). As such, the formation of inclusion complexes involves hydrophobic forces that facilitate the incorporation of a hydrophobic ligand component (e.g., the aliphatic fatty acid chain) into the amylose helical cavity (Godet, Tran, Delage, & Buléon, 1993).

Amylose/starch inclusion complexes are commonly prepared using one of three methods; high temperature, dimethyl sulfoxide (DMSO), or alkali (Putseys, Lamberts, & Delcour, 2010). The first step in all three methods is to obtain loose helices or random coils of amylose molecules by dissolution in water, DMSO or alkaline solutions. Next, the guest molecule is introduced into the amylose dispersion and, given the right conditions, inclusion complexes form, crystallize, and precipitate (Karkalas, Ma, Morrison, & Pethrick, 1995; Putseys, Lamberts, & Delcour, 2010).

The importance of amylose/starch-lipid inclusion complexes is reflected in numerous food applications, such as their anti-staling effect in baked goods (Fu, Chen, Luo, Liu, & Liu, 2015). The presence of either native lipids or added lipid emulsifiers can dramatically influence the pasting properties of starch, retard starch retrogradation, and extend the shelf life of baked goods. For instance, the functionality of saturated monoglycerides as crumb softeners in bread was attributed to their ability to form inclusion complexes with amylose (Riisom, Krog, & Eriksen, 1984). The amphiphilic nature and chemical structures of ARs are close to many lipid emulsifiers commonly added to bread. It was suggested that ARs may form inclusion complexes

with starch, as with other polar lipids during baking (Chen, Ross, Åman, & Kamal-Eldin, 2004), yet no study has confirmed this to our knowledge.

The objective of this study was to investigate the formation of starch inclusion complexes with ARs, which were extracted from rye bran, and the selective preference based on AR structure. Complementary techniques, including differential scanning calorimetry (DSC), X-ray diffraction (XRD), and Fourier transform infrared (FT-IR) spectroscopy, were used to confirm the formation of starch-ARs inclusion complexes. Liquid chromatography – mass spectroscopy (LC-MS) analysis was used to examine the AR profile in bran extracts and identify which ARs preferentially formed inclusion complexes with starch. The present work provides the foundation for the potential application of ARs as a "natural" and/or grain-derived anti-staling agent.

## 2. Materials and methods

### *2.1. Materials*

High amylose corn starch (HACS, Hylon VII) was kindly provided by Ingredion Inc. (Bridgewater, NJ). Acetone, ethanol, DMSO, diethyl ether, 2-propanol, and formic acid were of analytical grade, methanol was LC-MS grade, and all were obtained from VWR International (Radnor, PA). Rye bran (12 mesh, 1.41 mm particle size, grain originally from Canada or Germany) was kindly provided by Snavely's Mill, Inc. (Mill Hall, PA).

### *2.2. Alkylresorcinol (AR) extraction*

ARs were extracted from rye bran with acetone using a ratio of 1:40 (bran:acetone, w/v) by continuous mechanical shaking for 16-24 h at 22 °C, according to previously reported procedures (Gunenc, HadiNezhad, Tamburic-Ilincic, Mayer, & Hosseinian, 2013). The extracts were subsequently filtered using Whatman no. 42 filter paper. Acetone was completely evaporated at

60 °C *in vacuo* using a Rotavapor-RE 111 (Buchi, Switzerland). The crude extract was stored at -20 °C until further use.

*2.3. Formation of HACS-AR inclusion complex*

HACS (500 mg) was dissolved in 10 ml of 95% (v/v) DMSO aqueous solution and kept in a boiling water bath with constant stirring for at least 1 h. One (1) ml of preheated (90 °C) crude AR extract in DMSO (1%, w/v) was added to into the HACS dispersion. The mixture was held for 15 min at 90 °C, after which 25 ml of preheated (90 °C) deionized water was added with vigorous stirring and incubated for a further 15 min at 90 °C. The sample was allowed to cool at room temperature (22 °C) for 24 h. The precipitate was recovered by centrifugation (10,000 × g, 15 min), and washed three times with 50% (v/v) ethanol aqueous solution and once with 100% ethanol to remove uncomplexed guests. The resulting pellet was transferred to an aluminum dish and allowed to dry in a desiccator at room temperature. Dried samples were pulverized into fine powders and used for further analyses (Kong & Ziegler, 2013).

*2.4. Extraction of ARs from inclusion complex*

Samples were extracted twice to determine the identity of AR components entrapped in amylose helices during complex formation. First, samples were extracted with 2-propanol/water (3:1, v/v) in a boiling water bath (for 2 × 2h and 1 × 1 h) (Ross et al., 2003). However, this treatment was insufficient to remove all included AR components (see Discussion below). Following 2-propanol/water extraction, the remaining inclusion complexes were dissolved in hot DMSO (in boiling water bath above 90 °C) to release the guests, which were then recovered by liquid-liquid extraction using ethyl acetate:diethyl ether:cyclohexane (1:1:1, v/v/v). 2-Propanol/water and liquid-liquid extracts were analyzed by LC-MS to identify the guest compounds in the starch inclusion complexes.

*2.5. Differential scanning calorimetry (DSC)*

Dissolution temperatures and enthalpies of the putative inclusion complexes during heating were examined by a Thermal Advantage Q100 DSC (TA Instruments, New Castle, DE). Approximately 5 mg of dried sample were weighed into a 60 µL stainless steel DSC pan (Perkin-Elmer Instruments, Norwalk, CT), to which deionized water was added resulting in a 10% (w/v) dispersion. The hermetically-sealed pan was equilibrated to 20 °C, heated to 160 °C at 10 °C/min, cooled to 20 °C, and then reheated to 160 °C at 10 °C/min. An empty sample pan was used as a reference. Onset ($T_{onset}$) and peak ($T_{peak}$) temperatures and enthalpy (ΔH) of the endothermic transition were determined using the Universal Analysis software (Universal Analysis 2000 v.4.2E, TA Instruments-Waters LLC, New Castle, DE).

*2.6. Wide-angle X-ray diffraction (XRD)*

The XRD patterns of the crude AR extract and the putative inclusion complexes before and after 2-propanol/water extraction were obtained by a Rigaku MiniFlex II desktop X-ray diffractometer (Rigaku Americas Corporation, TX), operating at a current of 15mA and voltage of 30 kV. Dry powdered samples were exposed to Cu Kα radiation (0.154 nm) and scanned between 2θ = 4 and 30 ° at a scanning rate of 2 °/min with a step size of 0.01 °. Data were analyzed with Jade v.8 software (Material Data Inc., Livermore, CA).

*2.7. Fourier transform infrared (FTIR) spectroscopy*

FTIR analyses of crude rye bran AR extract, HACS-AR inclusion complexes before and after 2-propanol/water extraction were performed on a Bruker v70 spectrometer (Bruker Optics Ltd., Billerica, MA) equipped with an MVP-Pro$^{TM}$ Star Diamond attenuated total reflectance (ATR) accessory (Harrick Scientific Products, Inc., Pleasantville, NY). Spectra were obtained at room

147 temperature over the wave number range of 400-4000 $cm^{-1}$, with an accumulation of 100 scans
148 and a resolution of 6 $cm^{-1}$.

149 *2.8. Liquid chromatography-mass spectrometry (LC-MS) analysis*

150 AR homologs were identified by LC-MS according to previously reported methodology
151 (Knödler, Kaiser, Carle, & Schieber, 2008). The crude AR extract, the extracts obtained from 2-
152 propanol/water extraction and DMSO/liquid-liquid extraction of the starch-guest complexes
153 were dissolved in 90% (v/v) methanol aqueous solution and filtered over polytetrafluoroethylene
154 (PTFE) syringe tip filters (0.45 µm, 13 mm; Acrodisc™, Ann Arbor, MI). The LC system
155 consisted of a binary pumping system (Shimadzu LC-10ADvp) with high-pressure mixing and
156 sample introduction by means of Shimadzu SIL 10ADvp auto-sampler (Shimadzu Corporation,
157 Columbia, MD). A gradient mobile phase program chromatographically separated the AR
158 homologs on a ZORBAX Eclipse Plus C18 column (4.6 x 150 mm, 5 µm; Agilent Technologies,
159 Santa Clara, CA) at a flow rate of 0.2 mL/min and temperature of 35 °C. Solvent A consisted of
160 0.1% (v/v) formic acid in methanol, and solvent B consisted of 0.1% (v/v) formic acid in water.
161 The gradient program for solvent A was as follows: min 0, 90%; min 10, 100%; min 25, 100%;
162 min 35, 90% (Gunenc, HadiNezhad, Farah, Hashem, & Hosseinian, 2015).

163 Detection of each AR homolog was achieved using a Waters Quattro micro triple quadrupole
164 mass spectrometer (Waters Laboratory Informatics, Milford, MA) coupled to the LC. Mass
165 spectra were collected in negative-ion mode using electrospray ionization (ESI). The ESI
166 capillary spray was operated at 0.50 kV, with a cone source voltage of 60 V, source temperature
167 of 120 °C, and desolvation gas flow of 250 L/h. Selective ion monitoring mode was set to
168 monitor ions with *m/z* of 320 (C15:0), 348 (C17:0), 376 (C19:0), 404 (C21:0), 432 (C23:0), and
169 460 (C25:0) for saturated AR homologs; 344 (C17:2), 346 (C17:1), 372 (C19:2), 374 (C19:2),

400 (C21:2), 402 (C21:1), 428 (C23:2), 430 (C23:1), and 458 (C25:1) for unsaturated AR homologs (Gunenc, HadiNezhad, Farah, Hashem, & Hosseinian, 2015; Ross, Åman, & Kamal-Eldin, 2004). ARs were identified based on UV absorbance and molecular mass values.

## 3. Results and discussion

### *3.1. Characterization of crude AR extract*

A crude extract of ARs from rye bran was obtained by acetone extraction and characterized by DSC, XRD, FT-IR and LC-MS. The crude extract was collected as a brown viscous paste showing a melting range of 20-50 °C (determined by DSC), which is generally characteristic of a mixed lipids system (Tufvesson, Wahlgren, & Eliasson, 2003a), but lower than the melting points of most pure ARs (Reusch & Sadoff, 1979). The XRD pattern of the crude AR extract (Fig. 1a) revealed a single sharp peak at $2\theta = 21.27$ °, characteristic of the 110 plane of crystalline lipids (e.g., fatty acids) (Chevallier, Provost, Bourdet, Bouroukba, Petitjean, & Dirand, 1999), superimposed on a broad amorphous halo, suggesting a mixture of short-range orders within the AR constituents. The FTIR spectrum of crude AR extract (Fig. 2a) showed the presence of characteristic AR bands at 3353, 2917, 2850, 1740, 1700, 1600, 1465, 1150, and 720 $cm^{-1}$. The band at 3353 $cm^{-1}$ (not shown in Fig. 2) is characteristic of bonded OH groups by medium strength H-bonds. Bands at 2917 $cm^{-1}$ and 2850 $cm^{-1}$ were attributed to $CH_2$ stretching vibrations of the alkyl chains. Bands between 1400-1600 $cm^{-1}$ were assigned to aromatic ring deformations and those at 1150 $cm^{-1}$ and 720 $cm^{-1}$ assigned to phenol COH deformation and $CH_2$ rocking of the aliphatic tail, respectively (Ciesik, Koll, & Grdadolnik, 2006).

The crude AR extract from rye bran contained ca. 74% ARs (Table 1) as estimated by LC-MS (Fig. 3a). Thirteen different AR homologs (6 saturated and 7 unsaturated) were identified in the crude extract, which is in agreement with previous reports (Zarnowski & Suzuki, 2004). The

estimated abundance of AR homologs were in the following order: C19:0> C17:0> C21:0> C19:1> C21:1> C23:0> C17:1> C25:0> C23:1> C21:2> C19:2> C15:0 > C17:2. Three dominant AR homologs were C19:0, C17:0 and C21:0, also in agreement with other studies (Gliwa, Gunenc, Ames, Willmore, & Hosseinian, 2011; Ross, 2012a). The crude AR extract from rye bran may contain substances (e.g., triglycerides, phospholipids, sterols, and free fatty acids) other than the targeted ARs (da Cruz Francisco, Danielsson, Kozubek, & Dey, 2005). In the present study, a sizable peak observed at a retention time of 2.24 min was tentatively identified as free fatty acids (Gliwa, Gunenc, Ames, Willmore, & Hosseinian, 2011).

*3.2. Characterization of HACS-AR inclusion complex*

Complementary techniques were employed to determine if inclusion complex could be formed by HACS and ARs using the DMSO method. The dissociation of starch/amylose-lipid inclusion complex generally produces an endotherm on DSC thermogram with a peak temperature in the range from 80 to 120 °C, which depends mainly on the alkyl chain length of the guest lipid (Tufvesson, Wahlgren, & Eliasson, 2003a, 2003b). The dissociation enthalpy is proportional to the amount of guest lipids that have been complexed, which in turn depends mainly on lipid structure and complexation process that varies among studies. In this study, an endotherm with $T_{onset} = 91.1 \pm 1.7\ °C$, $T_{peak} = 104.2 \pm 2.1\ °C$, and an enthalpy of 20.1 ± 3.6 J/g during the first heating from 20 to 160 °C indicated the formation of HACS inclusion complex (Fig. 4a). HACS inclusion complex was seen to reform upon cooling after dissociation, as was evident during the second heating from 20 to 160 °C, wherein an endothermic peak at $T_{onset} = 89.5 \pm 2.1\ °C$, $T_{peak} = 101.4 \pm 1.2\ °C$, and an enthalpy of 13.3 ± 2.0 J/g was observed (Fig. 4b). The formation of HACS inclusion complex during cooling is a process similar to the high temperature method for starch inclusion complex formation. Complexed guest compounds

released after first heating could induce inclusion complexation with HACS/amylose molecules that were converted to loose helical or random coil conformations above 140 – 160 °C (Creek, Ziegler, & Runt, 2006). However, due to relatively rapid cooling and the lack of an annealing procedure, the extent of inclusion complexation could be lower as evidenced by a lower dissociation enthalpy during the second heating.

The XRD pattern of the HACS inclusion complex (Fig. 1b) contained a weak and broad peak at $2\theta$ = 8.28 °, suggesting the presence of poorly organized V-type crystals. This poor organization might be due to large diphenol head groups of ARs that cannot neatly pack, the varying length of alkyl chains, the presence of unsaturated fatty acid esters, or the likely combination of all these factors. Characteristic IR bands of ARs were also present in the FTIR spectrum of the HACS inclusion complex, though at a lower magnitude. Complementary DSC, XRD, and FTIR techniques confirmed the formation of HACS inclusion complex with constituents in crude AR extract, however, the complexation between HACS and non-AR components cannot be excluded.

*3.3. Characterization of ARs in the inclusion complex*

Non-AR components, e.g., free fatty acids, mono- and diacylglycerols (Gliwa, Gunenc, Ames, Willmore, & Hosseinian, 2011), in the crude AR extract from rye bran are also capable of forming inclusion complexes with HACS/amylose. To identify the guest compounds in the HACS inclusion complex, the sample was extracted with a hot (>90 °C) 2-propanol/water solution (3:1, v/v). It was assumed and expected that washing with ethanol during preparation of the inclusion complex had removed uncomplexed guest compounds (Kong & Ziegler, 2013), and the hot 2-propanol/water solution could extract complexed guest compounds. LC-MS analysis of the 2-propanol/water extract (Fig. 3b) revealed 13 AR homologs, in order of abundance C19:0>

C17:0> C21:0> C19:1> C23:0> C21:1> C17:1> C25:0> C23:1> C21:2> C15:0 > C19:2 > C17:2. The 2-propanol/water extract contained the same ARs (13 homologs) as the crude AR extract (Fig.3a). The uncharacterized peak observed at the beginning ($R_T$ = 2.24 min) of the LC-MS chromatograms (Fig. 3a and b) was assumed to be free fatty acids, which is well known to form inclusion complex with amylose (Wokadala, Ray, & Emmambux, 2012).

After the 2-propanol/water extraction, the sediment remained slightly brown, suggesting that not all of the complexed compounds were extracted. Thermal analysis by DSC again showed an endotherm characteristic of HACS/amylose inclusion complex (Fig. 5), but at a slightly higher temperature, suggesting that the crystal structure had been annealed (Putseys, Lamberts, & Delcour, 2010). Thermograms for the inclusion complexes after 2-propanol/water extraction showed endothermic peak temperatures at $T_{onset} = 100\ °C$, $T_{peak} = 111\ °C$, and enthalpy of 26 J/g for the first heating (Fig. 5a), and $T_{onset} = 90\ °C$, $T_{peak} = 104\ °C$, and enthalpy of 13.5 J/g for the second heating (Fig. 5b). The endotherm during second heating is again the dissociation of inclusion complex formed during cooling, and the onset and peak temperatures and enthalpy are close to those in the second heating of inclusion complex prior to extraction. The sample after 2-propanol/water extraction demonstrated a better resolved $V_{6h}$-type pattern with characteristic peaks at 2θ = 8.28, 13.11, and 19.93 ° (Fig. 1c), although these peaks were still weak compared with those from HACS inclusion complexes with pure fatty acids or their esters (Kong & Ziegler, 2013; Kong & Ziegler, 2014; Lay Ma, Floros, & Ziegler, 2011). This again suggested that while some complexed guest compounds were extracted, the remaining V-type semicrystalline inclusion complex was annealed during the extraction process. We have demonstrated that alcohol/water solutions could anneal the V-type HACS/amylose structures by

261 significantly increasing their crystallinity and the dissociation temperature of V-type
262 HACS/amylose inclusion complexes (Kong & Ziegler, 2014).

263 In order to extract the remaining guest compounds, the sample was disrupted in DMSO,
264 which is a good solvent to dissolve V-type HACS inclusion complexes. The guests were
265 recovered by liquid-liquid extraction using ethyl acetate:diethyl ether:cyclohexane. LC-MS
266 chromatograms (Fig. 3c) showed a decrease in the total number of AR homologs (9), of which
267 five were saturated and four were unsaturated (Table 1). The order of AR homolog abundance
268 from most to least was C21:0> C19:0> C23:0> C21:1> C19:2> C23:1> C17:0> C25:0> C19:1.
269 The most abundant AR homologs in crude AR extract were C19:0, C17:0 and C21:0, whereas
270 the most abundant ones extracted from the inclusion complex samples were C21:0 and C19:0.
271 The C15:0, C17:1, C17:2, and C21:2 AR homologs were not detected by LC-MS following the
272 second extraction as they could have all been removed during the 2-propanol/water extraction
273 step. These findings could be expected as longer hydrocarbon chains allow more hydrophobic
274 interactions with the hydrophobic helix cavity, and, therefore, would be less likely to be
275 extracted by modestly polar solvents like 2-propanol/water. In the formation of HACS/amylose-
276 lipid complexes, the lipid chain length, degree of unsaturation, and identity of the polar head
277 impact the complex properties (Putseys, Lamberts, & Delcour, 2010). The presence of carbon-
278 carbon double bond, especially in *cis* configuration, may interfere with amylose wrapping around
279 the alkyl chain and result in distorted helical element with lower stability. This is reflected from
280 the composition of the second extract where unsaturated AR homologs were in low quantity and
281 some were even undetectable. Free fatty acids, without the bulky phenolic polar head group in
282 ARs, were more easily to induce inclusion complexation with HACS/amylose. As a result, both
283 extracts of the inclusion complex sample showed higher fatty acid content than that in crude AR

extract. However, it is also noticed that the fatty acids were easier to be extracted from their HACS inclusion complex with hot 2-propanol/water since the first extraction yielded a higher percentage of these components (Fig. 3b and Table 1). This was supported by the difference in the relative intensity of IR absorption of carbonyl-containing species at 1710 $cm^{-1}$ and 1740 $cm^{-1}$ to that for ARs at 1600 (Fig. 2a vs. 2d). Therefore, it reiterated that HACS/amylose could preferentially form inclusion complex with certain ARs homologs over other homologs and even fatty acids.

## 4. Conclusions

In conclusion, we have demonstrated, for the first time, that rye bran ARs are capable of inducing inclusion complexation with HACS or specifically its amylose component. Complementary DSC, XRD, and FTIR techniques were employed to confirm the formation of HACS inclusion complex. The inclusion complex sample was subject to two step extractions followed by LC-MS analyses, which showed both ARs and free fatty acids were complexed and the preferred AR homologs were those with saturated long alkyl chains. Future studies are suggested to consider the relationship between ARs potential to form amylose inclusion complex that has been found in this study and their ability to retard starch retrogradation, as this may lead to potential applications of ARs as novel anti-staling agents in the food industry. As ARs are derived from grains, they could serve as attractive “natural” alternatives to certain conventional anti-staling agents.

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## Conflict of interest

There is no conflict of interest.

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