A Thesis for the Degree of Master of Engineering

Stability enhancement of black rice anthocyanin using cyclodextrin produced from rice starch

쌀전분 유래 환형덱스트린을 이용한 흑미 안토시아닌의 안정성 향상

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Abstract

Black rice is a food crop that contains a variety of useful substances, especially anthocyanins (ACNs) widely known as a representative natural antioxidant. However, application of black rice containing ACN has been limited as ACN belongs to one of the most unstable compounds among natural products, and its crystallization is difficult. Therefore, in this study, the improvement of ACN stability was attempted by using cyclodextrin (CD), a versatile host molecule capable of forming a complex with various guest molecules to improve their thermal and photo- stability. The optimization of CD production from various rice starch was also conducted and treated with isoamylase, CGTase from *Thermoanaerobacter sp*., and glucoamylase.

The physicochemical properties of five different cultivars of rice starch, Dodammi (DD), Ilpummi (IP), Saegoami (SG), Sintoheukmi (SH), and Geonganghongmi (GH) were investigated and the yields of CD production were compared. Due to the highest amylose content of 66%, DD showed significant differences from the other cultivars in the results of various physicochemical analyses such as HPAEC, RVA, DSC, XRD and SEM. From the preliminary experiments to determine the optimum conditions for CD production using SG

starch, the maximum yield of 76.64% was obtained when 5 U/g isoamylase was treated for 8 h, 25 U/g CGTase was treated for 6 h, and 50 U/g glucoamylase was treated for 6h. When CD was produced from the five cultivars at the optimal conditions, DD starch showed the highest yield of 82.91%. Therefore, DD was selected as the final substrate for CD production.

After extraction, ACN was pulverized by lyophilization. To investigate the effect of CD on ACN stability, 0-2% of β-CD was mixed with 0.1% ACN at pH 2, 4, 6, and 8 for 24 h, and a decrease of antioxidant activity during heat treatment (96℃) and UVB irradiation for 24 h was monitored using ABTS method. In the heat treatment condition, the addition of β-CD at pH 2 resulted in a significant improvement in antioxidative stability of ACN. A similar improvement of ACN antioxidative stability with β-CD was observed for UVB irradiation tests at pH 2. The degradation constant (kd) value and half-life ($t_{1/2}$) of antioxidant activity suggested that ACN existed in a structure susceptible to heat at pH 2 and vulnerable to UVB at pH 4, and CD worked to protect ACN at these pHs. As a result of comparing the antioxidative stability of ACN with the addition of CD produced from DD (DD-CD) and maltodextrin (MD) at pH 2 and 4 under the same conditions, the addition of DD-CD was superior to the addition of MD and β-CD. This result indicated that CD was superior to MD in complex formation with ACN, and α-, β-, and γ-CD contained in DD-CD were more effective to protect ACN than β-CD only.

In conclusion, this study confirmed that highly efficient CDs could be produced from rice starch by sequential treatment of isoamylase, CGTase, and glucoamylase. Also, CD was found to form a complex with ACN to alleviate pH-dependent

thermal and photo- instability of ACN and suggested the possibility of industrial application. However, in the production of DD-CD, the activity of CGTase was inhibited by sugar and CD. Therefore, a process of separating and adsorbing CD was required during the process. In addition, since ACN is known to be unstable in environments other than light or heat, further studies about the effect of DD-CD on the storage stability and oxidative stability of ACN will be needed. It is highly feasible that DD-CD contained stable antioxidant materials can be developed and also, the utilization of black rice can be expanded in the future.

Key words: Black rice, anthocyanin, cyclodextrin, isoamylase, cyclodextin glucanotransferase, glucoamylase, thermal stability, photo-stability

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Ⅰ. Introduction

Rice has been regarded as an important food resource in the world (Ichikawa et al., 2001). There are various cultivars of rice such as japonica rice, indica rice, flavored rice, and so on. Among them, black rice is especially known as economically important rice species due to its beneficial components. Black rice contains polyphenolics, vitamin E, Tocopherol, γ-oryzanol, and rich anthocyanin (ACN) (Kong & Lee, 2010). However, black rice is often used as a cooked rice form, but it is not utilized as a functional material because of its low stability of ACNs. Therefore, studies to improve the stability of the useful components of black rice are needed.

ACN, a group of phenolic compounds, is a glycosylated polyhydroxy and polymethoxy derivative of flavilium salts (Serraino et al., 2003). It is a representative functional material in black rice and exists in different chemical structures depending on the degree of acidity or alkalinity (Brouillard & Dubois, 1977). A notable characteristic of ACNs is antioxidant activity that is effective in the prevention of neurological and cardiovascular illnesses, cancer and diabetes, among others (Patras et al., 2010).

Despite the great potential of ACN, it is difficult to utilize in a variety of industries, since it is one of the most unstable compound among natural products and its degradation can be occurred by many factors, such as temperature, oxygen, light, and so on (Mourtzinos et al., 2008). Therefore, many studies were reported technologies of encapsulation of anthocyanin, including spray drying, coacervation,

liposome entrapment, inclusion complex, cocrystallization, nanoencapsulation, freeze drying, yeast encapsulation and emulsion to enhance the stability of ACN (Fang & Bhandari, 2010). Among these various technologies, inclusion complex using cyclodextrin (CD) was considered interesting, because CD was similar with amylose and amylopectin in which natural ACNs coexist (Yamada et al., 1980).

CD is a cyclic oligosaccharide most commonly consisted of 6, 7, or 8 glucosidic units named α-, β-, and γ-CD, respectively (Dodziuk, 2006). The most remarkable ability of CD is forming inclusion complexes with a variety of guest molecules due to their unique structure of hydrophobic cavity (Rymdén et al., 1983; Thaning et al., 2008). Among the α-, β-, and γ-CD, β-CD is especially useful in the industry due to its high encapsulation efficiency, appropriate cavity dimensions, and low cost (Sancho et al., 2011). Mourtzinos et al. (2008) protected ACNs from thermal degradation by the presence of β-CD. They reported that the inclusion complex of H. sabdariffa L. extract with β-CD was remained intact at 100-250 ℃ where the free extract was oxidized. Howard et al. (2013) also investigated the effect of β-CD against the stability of Chokeberry ACNs and identified that addition of 3% β-CD with chokeberry at pH3.6 showed excellent protection of ACNs with 81% retention after 8 months of storage at 25℃.

As mentioned above, studies on the interaction of ACN with CD have been reported, but no in-depth studies have been reported on the thermal- and photo-stability of CD and ACNs produced in rice. Thus, in this study, the degradation of antioxidant activity against heat and light was investigated by forming complexes of ACNs with β-CD, CD from rice starch, and maltodextrin (MD). Special rice

starches, Dodammi (DD), Ilpummi (IP), Saegoami (SG), Sintoheukmi (SH), and Geonganghongmi (GH), with high-amylose content were used as substrate for producing CD and ACN extracted from SH for the first time. The enzymatic processes for the efficient CD production were investigated using cyclodextin glucanotransferase (from Thermoanaerobacter sp., EC 2.4.1.19), isoamylase, and glucoamylase. Also, ACN was appeared with a degree of antioxidant activity by ABTS assay.

Therefore, the goals of this research were to develop enzymatic processes for the efficient CD production from rice starch and to enhance the stability of ACN by forming a complex with CD.

Specific objectives are as follow.

1. Identification of physicochemical characteristics of various rice cultivars.
2. Enzymatic production of CD and comparing the yield of CD produced from various rice cultivars.
3. Development of functional complex using CD with ACN and investigation of its effect on the stability of ACN.

Ⅱ. Background and literature review

2.1. Starch

Starch is widely used in many industries for a variety of purposes such as adding stability, gelling, thickening, or lowing cost (BeMiller & Whistler, 2009). Normal starches are composed of two polymers of glucose, amylose and amylopectin. They form insoluble and semicrystalline structure of starch granule with an internal lamellar structure (Zeeman et al., 2010). Amylose, a linear chains of α-1,4 linked glucose residues, can have a degree of polymerization (DP) as high as 600 and molecular weight of amylose is about 10^6 Da. Amylose can complex with free fatty acids, glycerides, some alcohols, and iodine through hydrophobic core due to hydrogen atoms of the internal lamellar structure. Amylopectin (with molecular weight ranging from 10^7 to 10^8 Da), a major component of starch, has α-1,4 linked glucose residues with about 5% of branch points and the molecule possesses three types of chains: A, B, and C chains (Fig. 1). A-chains, the outer chains of amylopectin, are linked with an inner chain (B) at latent reducing group by C_6 of a glucose residue. B-chains are longer than A-chains and carry one or more chains as branches. The single C-chain has the sole reducing terminal residue and other chains as branches like B-chains (Buléon et al., 1998).

Starch can be modified to increase its quality and value by chemical reaction, physical reaction, and enzymatic treatments (Bemiller, 1997). Enzymatic methods are commonly used because they are economical and efficient to produce modified starch (Satyanarayana et al., 2004). For these reasons, enzymes used in

modification of starch, such as amylases, isoamylases, glucanosyltransferases, and glucoamylases, are favoured and noteworthy (Ray, 2011).

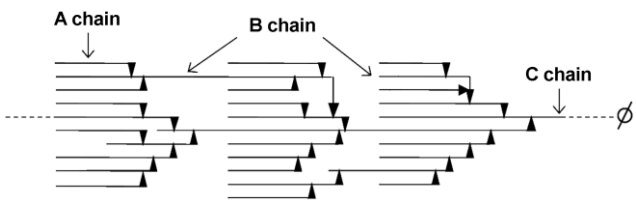


Fig. 1. Model of the different chains constituting the amylopectin.

2.2. Cyclodextrin glucanotransferase (EC 2.4.1.19)

Cyclodextrin glucanotransferase (EC.2.4.1.19; CGTase) is one of the important industrial enzymes isolated from more than 30 bacteria such as *Bacillus*, *Thermoanaerobacter*, *Klebsiella*, and *Actinomycetes*species (Qi & Zimmermann, 2005). Although CGTase is traditionally known as exo-acting enzyme producing cyclodextrin(CD), it has many other multiple functions (Lu & Xia, 2014). CGTase can catalyze transglycosylation reaction as well as hydrolysis reaction on starch and CDs (Tonkova, 1998). Such activities of CGTase on inter- and intramolecular transglycosylation of α-1,4-glucan are known as the cyclization reaction (Eq. 1-(1)) and the disproportionation reaction (Eq. 1-(2)), respectively. CGTase is also capable of catalyzing the transglycosidic linearization (coupling reaction), the reverse reaction of cyclization, of CDs with an acceptor molecule to produce linear α-1,4-glucans (Terada et al., 1997).

$$G_n \underset{\text{coupling}}{\overset{\text{cyclization}}{\rightleftharpoons}} G_{(n-x)} + cG_x \qquad (1)$$

$$G_n + G_m \underset{}{\overset{\text{disproportionation}}{\rightleftharpoons}} G_{(n-y)} + G_{(m+y)} \qquad (2)$$

Eq. 1. The reactions of CGTase (Schmid, 1989).

CGTase is usually classified as α-, β-, or γ-CGTase according to the type and yield of CDs produced at the highest ratios under the reaction conditions (Kamaruddin et al., 2005) (Table 1). Toruzyme 3.0 L, a popular commercial CGTase tefrom *Thermoanaerobacter sp.*, belongs to the β-CGTase family (Lu & Xia, 2014). Toruzyme 3.0 L produces a mixture of CDs with 6 (α), 7 (β), and 8 (γ) glucose units from the starch (Zhou et al., 2010). Initially, α- and β-CD are formed as equal levels, but if the reaction is continued, β-CD becomes the major element. Toruzyme 3.0 L also prefers using γ-CD as the donor in disproportionation reactions than producing it through cyclization reaction (Ara et al., 2015).

Most CGTases that have been investigated can produce more than one type of CDs as Toruzyme 3.0 L, because α-, β-, and γ-CD have a dimensionally distinct central cavity and different specificity for CGTases producing a specific type of CD. For this reason, recent studies have been focused on either rational protein engineering of CGTases based on the mechanism of the cyclization reaction or design of reaction methodology by optimizing the production with physico-biochemical parameters to use the wild-type enzyme for improving the yield of specific types of CDs (Terada et al., 1997).

Table 1. Type of CGTases produced from various bacteria (Schmid, 1989).

Organism	CGTase type	Gene cloned
Klebsiella oxytoca M5al	α	+
Bacillus macerans	α	+
Bacillus stearothermophilus	α	+
Bacillus circulans	β	+
Bacillus megaterium	β	-
Bacillus ohbensis	β	-
Micrococcus sp.	β	-
Alkalophilic Bacillus 38-2	β	+
Alkalophilic Bacillus 17-1	β	+
Alkalophilic Bacillus 1011	β	+
Alkalophilic Bacillus 1-1	β	+
Bacillus subilis No. 313	γ	+
Alkalophilic Bacillus 290-3	γ	+

2.3. Cyclodextrin (CD)

2.3.1. Properties and structures

Cyclodextrins (CDs) are cyclic and nonreducing oligosaccharide composed of α-D-glucose units with α-1,4-glucosidic linkages produced from starch or starch derivates using CGTase (Szerman et al., 2007; Biwer & Heinzle, 2004). CDs are designated by the number of glucose units: α (6 units)-, β (7 units)-, γ (8 units)-CDs and so on (Fig. 2). The three major CDs, α-, β-, γ-CD, are crystalline, homogeneous, nonhydroscopic substances which have a torus like macroring shape. Each CD has different diameter and solubility due to the different number of glucose units (Table 2). α-CD has the smallest molecular weight and diameter, but water solubility is higher than β-CD. The water solubility of β-CD at room temperature (25℃) is 1.79%, the lowest solubility of all the major CDs, because the C2-OH group of one glucopyranoside unit can build a hydrogen bond with the C3-OH group of the next glucopyranose unit and these hydrogen bondings form a complete secondary belt, making it an inflexible structure in the cavity of β-CD. On the other hands, the α-CD has the incomplete hydrogen bond belt, as one glucopyranose unit is in a distorted position. The γ-CD is more flexible structure compared to the other CDs, and is therefore more soluble than α- and β-CD (Frömming & Szejtli, 1993).

The important property of CDs is forming inclusion complexes with various hydrophobic and insoluble low molecular weight-compounds by its unique hydrophobic interior cavity (Kim & Lee, 1997). The cavity is lined by the

glycosidic oxygen bridges and the hydrogen atoms, respectively. Due to the structure of hydrophobic cavity, diverse properties of the guest molecules, such as their water solubility, stability, and bioavailability can be efficiently manipulated (Kaulpiboon & Hansakul, 2007). For this reason, CDs are one of the most widely utilized materials in food, cosmetic, pharmaceutical and any other industries.

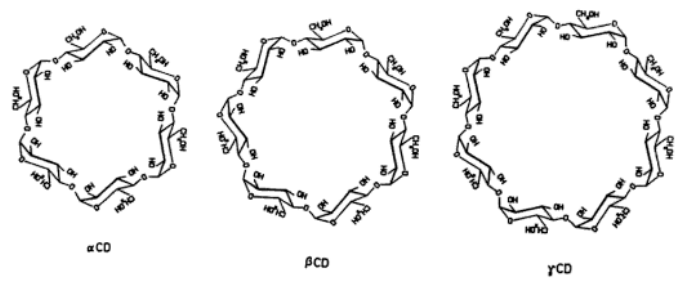


Fig. 2. Structure of α-, β-, and γ-CD (Frömming, 1993).

Table 2. Solubility of α-, β-, and γ-CD with different condition (Li & Purdy, 1992).

characteristics	**α**	**β**	**γ**
no. of glucose units	6	7	8
Molecular weight	972	1135	1297
Solubility in water (g/100mL)	14.5	1.85	23.2
Cavity diameter (Å)	4.7 - 5.3	6.0 - 6.5	7.5 – 8.3
Height of torus (Å)	7.9 ± 0.1	7.9 ± 0.1	7.9 ± 0.1
pK_a values	12.33	12.20	12.08

2.3.2. Applications

Szejtli (1988) reported CDs can protect active ingredients against oxidation, light-induced reactions, heat-promoted decomposition, and loss by volatility or sublimation in food. Also, CDs can eliminate or reduce undesired tastes/odors, microbiological contaminations, fibres/other undesired components, hygroscopicity, etc. Szente & Szejtli (2004) found that various CD complexes can be used in foods as conserving agents or antiseptic, 0.1% iodine- β-CD inhibits putrefying for 2 months as 20℃ in fish paste or in frozen sea-food products. In drug delivery, Loftsson et al. (2005) reported that CD is utilized to increase aqueous solubility and stability of poorly soluble drugs, and to improve drug delivery so that the complexing agents increase their bioavailability. CD is also useful in peptide, protein, and oligonucleotide delivery (Irie & Uekama, 1999) as well as a gene delivery system (Li & Loh, 2008).

2.3.3. Production

Szerman et al. (2007) reported that different types of starch can be used as substrate, but starch containing a high percentage of amylose such as maize starch and wheat starch gives lower yields of CD than amylopectin. They used cassava starch that has a high amylopectin content and obtained 66% (w/w) yield of CD with 1.00:0.70:0.16 of the α-CD: β-CD: γ-CD weight ratio. Sago starch, obtained from the cycad *Cycas revolute* synthesized 25.97 g/L of β-CD (the distributions of α-, β-, and γ-CDs were 7%, 65%, and 28%, respectively) using partially purified

Bacillus circulans CGTase by ammonium sulfate precipitation at 50–70% saturation (Charoenlap et al., 2004).

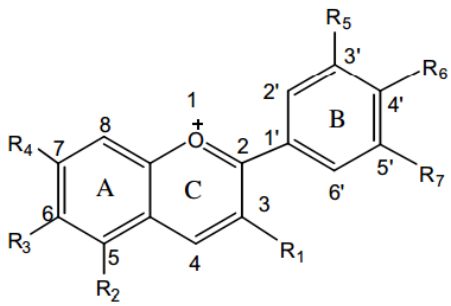
A preliminary treatment of starch by physical, chemical, and enzymatic methods is recommended when starch is used as raw material for CD production, because the high viscosity of the medium interrupts stirring and contact between the enzyme and the substrate to be hydrolyzed (Biwer & Heinzle 2004). Moreover, CGTases do not act on the α-1,6-linkages of amylopectin and a part of the starch molecule remains unused, so preliminary treatment of starch with debranching enzymes like pullulanase or isoamylase which cleave α-1,6- linkages is needed to make this part of starch chain accessible for CGTases (Rendleman, 1997). For instance, Duan et al. (2013) developed a simultaneous conversion approach by conjunction use of isoamylase from *Thermobifida fusca* with α-CGTase, and the total yield of CD on the optimum condition reached 84.6 % (w/w) after 24 h, which was 31.2% higher than transformation with α-CGTase alone. Also, Pishtiyski et al. (2006) found that CD yield was 50.9% by using α-amylase, and the effect of debranching was highest in the case of corn starch: the CD yield increased by 10%. Without pretreatment, the yield of CD and maltodextrin was 27.9 and 31.4%, respectively, prior to removal of 40.7% of residual corn starch by centrtfugation (Kim et al., 1997).

2.4. Anthocyanin (ACN)

Anthocyanins (ACNs) are pigments of flavonoid group that widely exist in many plant and fruits as red to purple to blue colors. ACNs can be used in food, cosmetic and pharmaceutical industries because they have great potential as natural colorants, antioxidant ability and also reducing the risk of cardiac diseases or cancer. However, ACNs are very sensitive to temperature, pH, light, oxygen and another factor, so their stability is quite low to processing and storage (Tonon et al., 2010).

The basic structure of ACNs are the anthocyanidins (or aglycons), consist of three bonded aromatic rings (Table 3); an aromatic ring [A], a heterocyclic ring [C] that contains oxygen, and third aromatic ring [B] (Konczak & Zhang, 2004). Anthocyanidins are known as ACNs when they are found in a glycoside form (bound to a sugar moiety) (Castaneda-Ovando et al., 2009). ACNs may exist in a variety of forms like protonated (flavylium cation; AH+), deprotonated (quinoidal base), hydrated (hemiketal), and isomeric forms (chalcone), and these molecules exist as the relative proportion that strongly depends on pH. These forms may play an important role in the antioxidant actions of anthocyanins. The completely conjugated structures of ACNs allowing electron delocalization results in very stable radical products, which is favorable considering antioxidativite ability (Kähkönen & Heinonen, 2003).

Table 3. Identification of anthocyanidins (aglycons) (Castaneda-Ovando et al., 2009).



General anthocyanins structure

Name	Abbreviations	Substitution pattern							Colour
		R_1	R_2	R_3	R_4	R_5	R_6	R_7	
Apigeninidin	Ap	H	OH	H	OH	H	OH	H	
Arrabidin	Ab	H	H	OH	OH	H	OH	OMe	N.R.[a]
Aurantinidin	Au	OH	OH	OH	OH	H	OH	H	
Capensinidin	Cp	OH	OMe	H	OH	OMe	OH	OMe	Blue-red
Carajurin	Cj	H	H	OH	OH	H	OMe	OMe	N.R.[a]
Cyanidin	Cy	OH	OH	H	OH	OH	OH	H	Orange-red
Delphinidin	Dp	OH	OH	H	OH	OH	OH	OH	Blue-red
Europinidin	Eu	OH	OMe	H	OH	OMe	OH	OH	Blue-red
Hirsutidin	Hs	OH	OH	H	OMe	OMe	OH	OMe	Blue-red
3'-HydroxyAb	3'OHAb	H	H	OH	OH	OH	OH	OMe	N.R.[a]
6-HydroxyCy	6OHCy	OH	OH	OH	OH	OH	OH	OH	Red
6-HydroxyDp	6OHDp	OH	OH	OH	OH	OH	OH	OH	Blue-red
6-HydroxyPg	6OHPg	OH	OH	OH	OH	H	OH	H	N.R.[a]
Luteolin	Lt	H	OH	H	OH	OH	OH	H	
Malvidin	Mv	OH	OH	H	OH	OMe	OH	OMe	Blue-red
5-MethylCy	5-MCy	OH	OMe	H	OH	OH	OH	H	Orange-red
Pelargonidin	Pg	OH	OH	H	OH	H	OH	H	
Peonidin	Pn	OH	OH	H	OH	OMe	OH	H	Orange-red
Petunidin	Pt	OH	OH	H	OH	OMe	OH	OH	Blue-red
Pulchellidin	Pl	OH	OMe	H	OH	OH	OH	OH	Blue-red
Riccionidin A	RiA	OH	H	OH	OH	H	OH	H	N.R.[a]
Rosinidin	Rs	OH	OH	H	OMe	OMe	OH	H	Red
Tricetinidin	Tr	H	OH	H	OH	OH	OH	OH	Red

[a] N.R: not reported.

Ⅲ. Materials and methods

3.1. Materials

Five Korean rice cultivars, Dodammi (DD), Ilpummi (IP), Saegoami (SG), Sintoheukmi (SH), and Geonganghongmi (GH) were acquired from Rural Development Administration of Korea. α–Cyclodextrins (α–CD, ≥ 98.0% purity), β–cyclodextrins (β–CD, minimum 98.0% purity), and γ–cyclodextrins (γ–CD, ≥ 98.0% purity) were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA) and used as a standard. Toruzyme® 3.0 L (CGTase, *Thermoanaerobacter* sp.) was provided from Novo Nordisk A/S (Denmark) to produce CDs. Isoamylase from *Pseudomonas* sp. and glucoamylase from *Hormoconis resinae*. were purchased from Megazyme (Wicklow, Ireland). Anthocyanin (ACN) was extracted from black rice, SH and ABTS (2, 2'-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid); A1888) was purchased from Sigma-Aldrich Co. (St. Louis, MO, USA). Maltodextrin (MD, Avg. M.W. = 2,160 Da (8-10 DE)) was obtained from Samyang Genex Co. (Seoul, South Korea). Any other reagents were bought from Junsei Chemical Co. (Tokyo, Japan), Duksan Pure Chemical Co. Ltd. (Kyungki, Korea) and so on.

3.2. Cyclodextrin (CD) production from various rice cultivars

3.2.1. Isolation of starches from various rice cultivars

Rice starches were isolated from 5 native rice cultivars (DD, IP, SG, SH, and GH, Korea) by the modified method of alkaline extraction (Yamamoto et al., 1973). Rice was washed and steeped in 2 volumes of deionized water at 25℃ for 3h to soften the endosperms. After then, the liquor was drained off and the endosperms were ground in successive small fractions with 5-6 volumes of 0.2% v/v sodium hydroxide solution using a blender. The slurry was sequentially passed through 45, 100, and 270 wire mesh sieves and the final starch suspension passed through 270 mesh allowed to be settled overnight. The cloudy supernatant was discarded, and the sediment was diluted to the original volume with sodium hydroxide solution. The process was repeated until the supernatant becomes clear and gives a negative reaction to the biuret test for protein. Starch was washed three times with distilled water, and then neutralized by 1 N hydrochloric acid solution. The sedimented starch was collected and dried at room temperature. The dried starch was ground using the blender and passed through 100 mesh.

3.2.2. Physicochemical properties of rice starches

3.2.2.1. Moisture content and apparent amylose content

The moisture content was measured in accordance with the AOAC method (1984). The samples were weighted 2 g and measured for 30 min using a moisture balance (Precisa HA 300).

The apparent amylose content of starch was measured with iodine colorimetry method by Williams et al. (1970) and Juliano et al. (1981). The 0.1 g of starches were suspended in the solutioncontaining 1 ml 99% (v/v) ethanol and 9 ml 1 N NaOH and leaved at room temperature for 24 h for dispersion. After that diluted with deionized water was added to 100 ml. 5 ml of suspension was decanted into 100 ml flask and 50 ml deionized water and 1 ml iodine solution were added at the same time. Finally, the volume was made up to 100 ml with deionized water. 5ml of 0.09 N NaOH solution was decanted into another flask and used as a blank. The results were measured using the UV–Vis spectrophotometer (UV-1650PC, SHIMADZU Co., Japan) at 620 nm. These experiments were performed in triplicates.

3.2.2.2. Rapid Visco-Analyzer (RVA)

The viscoamylographs of the starches were measured by using a Rapid Visco-Analyzer model RVA4 (RVA) (Nowport Scientific Pty, Ltd., Wariiewood, Australia) in accordance with the AACC method 61-02 (1995). The sample starch 3 g and 25 ml of distilled water were mixed in the sample canister. Pasting

condition was maintained for 1 min at the initial temperature 50℃ and then held at 95℃ for 4 min. Finally, it was maintained for 4 minutes after the temperature was cooled to 50℃. The RVA pasting parameters of peak viscosity, trough viscosity, final viscosity, break down (peak-trough), setback (final-trough), peak time and pasting temperature were calculated. Viscosity units were marked as Rapid Viscosity Unit (RVU).

3.2.2.3. High-performance size exclusion chromatography (HPSEC)

1% (w/v) of starches dissolved in 90% (v/v) DMSO were boiled with stirring for 30 minutes and continuously stirred for 24 h at room temperature. After that, the 5-fold volume of ethanol was added into the starch solutions and centrifugated (10000 × g, 20 min) to collect the precipitated starches. In the same way as above, 3-fold volume of acetone was added into the precipitated starches for centrifugation and then, dried in hood.

The samples were analyzed with HPSEC (high performance size exclusion chromatography) combined with a refractive index detector. The HPSEC system used running columns (OH-Pak 804, OH-806 HQ, Shodex) at room temperature. The flow rate of mobile phase (pure water) was 0.4 ml/min. The molecular weight was measured with the pullulans standards.

3.2.2.4. High-performance anion exchange chromatography with pulsed amperometric detection (HPAEC-PAD)

Distribution of branched amylopectin chain length was analyzed by high performance anion exchange chromatography-pulsed amperometric detection (HPAEC-PAD). The sample starches were treated as above HPSEC sample method. Then, 5 mg of powders were dissolved in 1 ml of 50 mM sodium acetate (pH 4.5) by boiling for 15 min. Isoamylase (1 U/mg) was added in the starch solutions at 40℃ for 2 h. In order to stop the reaction, the starch solutions were boiled for 10 min. The samples were filtered using 0.45㎛ filter (disposable membrane, nylon) and injected into HPAEC.

HPAEC with a Dionex DX-300 (Dionex Cor., Sunnnyvale, CA, USA) system and pulsed amperometric detector (ED40, Dionex) used CarboPac TM PA-1 anion-exchange column (250 × 4 mm, Dionex, USA). The column was equilibrated with 150 mM NaOH and the samples were eluted with varied gradients of 600 mM sodium acetate in 150 mM NaOH at a flow rate of 1 ml/min. The solvent gradient condition of the analysis of the distribution of branch-chain length was summarized in Table 4.

Table 4. HPAEC solvent gradient condition for the chain length distribution of starches.

Time (min)	**0**	**10**	**16**	**27**	**44**	**63**	**70**	**70.10**	**75.01**	**80.01**
Buffer A[a)] (%)	90	70	60	50	40	35	34	0	100	90
Buffer B[b)] (%)	10	30	40	50	60	65	66	100	0	10

a) 150 mM NaOH in water.

b) 600 mM sodium acetate in 150 mM NaOH.

3.2.2.5. X-ray diffractometry (XRD)

The degree of crystallinity of rice starch granules were analyzed by powder X-ray diffractometry (XRD, D8 Advance, Bruker AXS GmbH, Karlsruhe, Germany) following the method of Nara & Komiya (1983). The starch powder samples were scanned using Cu Kα radiation (λ = 1.54056 Å) at 40 kV and 30 mA. The scanning region of the angles (2θ) was from 5° to 35° at a scanning speed of 0.04°/ min.

3.2.2.6. Differential scanning calorimeter (DSC)

The thermal properties of starch samples were measured and recorded on a differential scanning calorimeter (DSC, Unix DSC 7, Parkin-Elmer Co., USA). The samples (10 mg) were added in the DSC pans as 1:3 starch-to-water ratios with distilled water (30 µl). The pans were sealed and equilibrated for 2-3 h at room temperature. The scanning temperature range was 20-90℃ and the heating rate was 5℃/min. The results were recorded with an empty pan as a reference. The onset of gelatinization (T_o), the peak temperature (T_p), the gelatinization temperature at conclusion (T_c), and melting enthalpy (ΔH) were calculated from endothermic peak by Lund method (1984). All experiments were performed at least three times.

3.2.2.7. Scanning electron microscopy (SEM)

Granule morphology was imaged by using scanning electron microscopy (SEM). The dried starch samples were mounted on circular aluminum boards with double-side tape and then coated with gold at 20 mA for 90 sec. Subsequently the morphology of samples was photographed with JSM-6700F field emission scanning electron microscopy (FE-SEM, Helios NanoLab 650).

3.2.3. CD production from various rice cultivars

3.2.3.1. Assay of CGTase activity

Cyclization activity of CGTase was determined by the phenolphthalein method with slightly modification (Kaneko et al., 1987; Mahat et al., 2004). 40 mg of soluble starch was mixed in 1.0 ml of 0.1M sodium phosphate buffer (pH 6.0). Crude enzyme 0.1 ml was added in soluble starch solution and incubated at 60℃ for 10 min. Reaction was stopped by adding 3.5 ml of 30 mM NaOH and 0.5 ml of 0.02% (w/v) phenolphthalein in 5 mM Na_2CO_3 solution. Then the reduction of colour intensity was measured at 550 nm by spectrophotometer. One unit of the enzyme activity was defined as the amount of enzyme forming 1 μmol of β-CD per min. Standard curve was plotted with β-CD concentrations (40-400 μg/ml).

3.2.3.2. Debranching of rice starch using Pseudomonas isoamylase

In order to optimize the reaction of starch debranching, SG starch was debranched with isoamylase (EC 3.2.1.68) from *Pseudomonas* sp. (Megazyme International Ireland). At first, the slurry of 1% starch dissolved in sodium acetate buffer (50 mM, pH 4.5) was boiled for 30 min. with stirring to gelatinize. After that, the gelatinized starch was cooled down to 40℃ and incubated with 5 U/g of isoamylase in a 40℃ water bath up to 15 h with stirring. In order to stop the debranching reaction, the enzyme-treated samples were boiled for 10 min and collected by precipitating with 10-volume of 95% ethanol.

3.2.3.3. Reducing sugar measurement

The reducing sugars were measured by dinitrosalicylic colorimetric (DNS) method (Miller, 1959) to estimate the degree of enzymatic hydrolysis. Each Aliquots of the liquid fraction 0.1 ml after isoamylase treatment for different reaction time are place into test tubes and 1ml of DNS reagent was added; 10.6 g of DNS, 19.8 g of sodium hydroxide, 306 g of Rochelle salts (sodium potassium tartrate), 7.6 mL of phenol crystals melted at 50℃, and 8.3 g of sodium metabisulfite were dissolved in 1416 mL of distilled water. To start the reaction, the mixture was boiled for 5 min and the reaction rapidly stopped by transferring it to ice. After 15 min, the mixture was left to room temperature for 5 min. The absorbance of the mixture was measured at 575 nm using UV–Vis spectrophotometer (UV-1650PC, SHIMADZU co., Japan). The amount of reducing

sugar was indicated as the average concentration of the three replicas calibrated by the standard curve.

3.2.3.4. Optimization of CGTase and glucoamylase treatment

SG starch (1% w/v) was suspended in 50mM sodium acetate buffer (pH 4.5) and debranched with 5 U/g of isoamylase at 40℃ for 8h with the same method described in the section 3.3.4.2. The debranched starch (1% w/v) was dissolved in 90% (v/v) DMSO and boiled for 30 min. After that, 100mM sodium acetate buffer (pH 6) was added and CGTase (10, 25 U/g) were treated. The reaction mixture was incubated at 60℃ up to 24 h. The enzyme reaction was terminated by heating the samples at 100℃ for 20 min. In order to purify and obtain the yield of CDs with HPAEC-PAD, glucoamylase from *Hormoconis resinae* was used. The pH of the CGTase-treated samples was adjusted to pH 4.5 by adding 1N acetic acid. The resulting solution was incubated with 50 U/g of glucoamylase up to 24 h at 40℃. At the end of the reaction, the enzyme of samples was inactivated by heating at 100℃ for 15 min.

3.2.3.5. Quantification of CDs by HPAEC-PAD

The yield of produced CDs was calculated through the peak areas using the same device and column described in the section 3.2.2.4. Several programs of solvent gradient were examined and the most suitable program to separate CDs was summarized in Table 5.

The standard curve of CDs was fitted using the peak areas of pure α-, β-, and γ-CDs. The pure CDs (1% w/v) were dissolved in distilled water and serial diluted up to 1.563×10^{-2} %. The standard curve had a linear range of 1.563×10^{-2}% to 2.5×10^{-2}%.

Table 5. HPAEC solvent gradient condition for the analysis of the chain length distribution of CDs.

Time (min)	**0**	**10**	**16**	**27**	**27.01**	**32**	**32.01**	**42**
Buffer A[a] (%)	90	70	60	50	0	0	90	90
Buffer B[b] (%)	10	30	40	50	100	100	10	10

a) 150 mM NaOH in water.

b) 600 mM sodium acetate in 150 mM NaOH.

3.2.3.6. Matrix assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS)

The molecular mass of produced CD was determined using a MALDI-TOF MS (Voyager^{TM}-DE, Perceptive Biosystem, Framingham, USA). The sample was mixed with a matrix (2,5-dihydroxybenzoic acid, 0.1-5 ρmol/μL), and the mixture was spread on the probe tip and dried until homogeneous crystals formed. The sample on the probe tip was desorbed/ionized under the appropriate conditions (Grid voltage, 87; Grid wire voltage, 0.3; Delayed extraction, 300nsec; and Laser, 2000). According to the assumption that a molecular weight is 162 g/glucose unit, theoretical masses of CD were calculated from $(C_6H_{10}O_5)_n$ and n is the number of D-glucose unit. The masses of non-cyclic glucans were calculated as 162n plus 18Da, which is the additional mass of a reducing end residue (Koizumi et al., 1999).

3.3. Interaction between anthocyanin and CD produced from high amylose rice starch

3.3.1. Extraction of ACN from black rice

ACN was extracted from black rice, SH with modified method of Hou et al., (2013). SH was ground using a blender and stored at -20℃. SH powder (5 g) was weighed and extracted with 60% v/v aqueous ethanol containing 0.2% v/v hydrogen chloride of solid-liquid ratio (1:10) for 90min at 50℃, 100 rpm. After then, the extracted solution was centrifuged for 15min with 4,000 rpm and the supernatant was filtered (0.45-μm pore size). The filtered ACN solution was used as lyophilized powder form to remove ethanol.

3.3.2. ABTS Antioxidant activity assay

ABTS assay was used to evaluate the antioxidant ability of ACN according to pH and CD concentration. There were some methods to determine antioxidant activity (Table 6), but each method has its own advantages and disadvantages (Yoo et al., 2007). In this study, ABTS method was chosen by its advantages of fast reaction and stability to pH, while DPPH method commonly used to measure antioxidant activity is sensitive to pH (Table 7) and its reaction time is slow.

7.4 mM ABTS and 2.6 mM Potassium persulphate were mixed 1: 1 and allowed to stand in a dark place for 24 h to form ABTS cations. After diluting ABTS solution with distilled water at 734 nm with absorbance of 0.7 degree, 20 μL of

samples and 980 μL of diluted ABTS solution were mixed. The mixture reacted for 20 min was measured its absorbance at 734 nm.

The antioxidant activity was calculated as the absorbance of the ABTS solution reduced by ACN versus the absorbance of the diluted ABTS solution in % unit (Eq. 2).

Eq. 2. Calculation of antioxidant activity

Antioxidant activity (%) = (Ablank-Asample) / Ablank x 100

Table 6. Methods to determine antioxidant activity *in vitro* (Yoo et al., 2007).

Method	Advantages	Disadvantages
ABTS	Applicable to both aqueous and organic phase, fast reaction	Very sensitive with temperature and light
	Using standard as vitamin C, hence can be expressed on a weight basis as natural source	Extra step to thermally generate free radical from ABTS salt
	Stable to pH hence can be used to study pH effect on activity	Not standardized, hence hard to compare values across laboratories
DPPH	Simple to use and inexpensive	Dissolved in organic solvents
	Reaching steady state quickly	Sensitive pH and slow reaction
	Good repeatability	Color interference causing underestimation of antioxidant activity
ORAC	To uses biologically relevant free radicals	Using standard as Trolox, hence cannot be expressed on a weight
	To integrate both degree and time of antioxidant reactionbasis as natural source	
	Various free radical generators can be used	
FRAP	Being expressed in ascorbic acid equivalents	Oxygen electrode, which may not maintain stable during measurement
	Easy and inexpensive	SH-group containing antioxidant are not detected

Table 7. The absorbance of DPPH solution as different pH levels.

pH	1.85	2.90	4.15	5.09	6.00	6.54
Abs.	0.49±0.03	0.57±0.03	0.58±0.03	0.59±0.02	0.59±0.01	0.62±0.01

3.3.3. Interaction of ACN with CD

3.3.3.1. Production of CD

1% (w/v) of DD starch was debranched with same methods as the section 3.2.3.2. The 5% (w/v) of debranched DD starch was suspended in 100 mM sodium acetate buffer (pH 6) and boiled for 30 min to gelatinize. After that, CGTase (5-50 U/g) was added and incubated at 60℃ up to 24 h. The enzyme was inactivated by heating the samples at 100℃ for 20 min. In order to purify and obtain the yield of CDs with HPAEC, 1 ml of CGTase-treated sample was diluted with 49 ml of 50 mM sodium acetate buffer (pH 4.5) and 0.5 U/g of glucoamylase from *Hormoconis resinae* was treated for 6h at 40℃. At the end of the reaction, the enzyme of samples was inactivated by heating at 100℃ for 15 min. The CD mixture from DD starch (DD-CD) was lyophilized to use as powder.

3.3.3.2. Fading effect of ACN with CD

In order to confirm the effect of pH and CD with ACN, the color change of ACN was investigated with slight modification as described by Fernandes et al. (2013). 0.2% (w/v) of ACN, lyophilized powder of extracted solution, was dissolved in universal buffer adjusted pH 2-5 and filtered with 0.45 μm pore sized filter. After then, the ACN solution was stirred with β-CD at an approximate volume ratio of the 1:0, 0.5, and 20 for 2 h in dark place and the spectrum of the samples was measured by UV-Vis spectrophotometer (UV-1650PC, SHIMADZU co., Japan).

3.3.4. Analysis of stability of ACN with CD

3.3.4.1. Thermal stability

Thermal stability of ACN was measured as a degradation of antioxidant activity with ABTS assay reported at section 3.3.2. 0.2% (w/v) of ACN powder was dissolved in universal buffer adjusted pH 2-6 and filtered with 0.45 μm pore sized filter. 0 - 4% (w/v) of β-CD, DD-CD, and MD were dissolved in distilled water and left under stirring with the 0.2% of ACN solution, respectively at volume ratio of 1:1 for 24 h at room temperature in dark place. After 24 h, aliquots of 500 μl of the samples were placed into Eppendorf tubes with brown color and heated up to 24 h at 92℃ using heat block (BF-20HB, Biofree, Korea).

3.3.4.2. Photo-stability

The complexes of ACN with β-CD, DD-CD, and MD were prepared as same methods of section 3.3.4.1. Aliquots of 1 ml of the complexes were placed into 20 ml vial and UVB irradiated. UVB irradiation was conducted in UV-chamber (40 × 35 cm) with 8 W UVB lamp (Sankyo Denki Co. Ltd., Japan, G8T5E) up to 24 h.

3.3.4.3. Degradation rate constant (k_d) and half-life ($t_{1/2}$)

The degradation rate constant (k_d) and half-life ($t_{1/2}$) were determined using thermal and photo-stability experiments. The degradation rate constant (k_d) was obtained by using Eq. 3-(1) of simple first-order kinetics (Microsoft Excel) and half-life ($t_{1/2}$) by calculating Eq. 3-(2). Where C_t is the ABTS radical scavenging activity of ACN (%) remaining in solution at time t (h), C_0 is the initial ABTS radical scavenging activity of ACN (t=0) and k_d is the rate of degradation constants (h^{-1}).

Eq. 3. Formula of the degradation rate constant (k_d) and half-life ($t_{1/2}$) of ACN.

(1) $C_t = C_0 e^{-k} d^t$

(2) $t_{1/2} = \ln 2 / k_d$

3.3.5. Statistical analysis

All data were performed duplicate of triplicate and recorded as mean ± standard deviation. For statistical analysis, the one-way ANOVA test followed by Tukey's *post-hoc* tests were performed using SPSS statistical software (SPSS version 23.0; SPSS Inc., Chicago, IL). In order to indicate a statistically significant result, a *p*-value < 0.05 was considered.

Ⅳ. Results and discussions

4.1. CD production from various rice cultivars

4.1.1. Physicochemical properties of rice starches

4.1.1.1. Moisture content and apparent amylose content

Moisture content of rice starches was reported 7.5-13.00% and Dodammi (DD) was indicated the highest moisture content (13.00%). Apparent amylose content was determined by considering moisture content (Table 8). The amoylose content of Saegoami (SG) was 31.26-34.00% almost same as 33.5-37.1% (Mira et al., 2013). Ilpummi (IP), Sintoheukmi (SH), and Geonganghongmi (GH) were also similar as previous study (Lee et al., 2013). DD was indicated to have higher apparent amylose content (66.76-68.89%) than previous report (42.8%) of National Institute of Crop Science in 2013.

Table 8. Moisture content and apparent amylose content of rice starches.

Rice Starch	Moisture content (%)	Apparent amylose content (%)	
		Williams's method	Juliano's method
DD	13.00	68.89±1.35	66.76±0.08
IP	10.84	23.40±0.38	20.69±0.26
SG	7.50	31.26±0.90	34.00±0.35
SH	9.00	21.61±1.40	18.38±1.06
GH	8.50	23.92±0.02	21.78±1.01

4.1.1.2. Pasting properties by RVA

Pasting properties of rice starches were analyzed by RVA. Viscogram and pasting viscosity index were respectively reported in Fig. 3 and Table 9. DD showed significantly lower characteristics of past viscosity and peak viscosity (73.67 cP) compared to other cultivars. As swelling of the starch was caused by amylopectin and inhibited by amylose, DD with the highest-amylose content exhibited the lowest peak viscosity because the swelling was the most strongly inhibited than the other cultivars. In the case of pasting temperature, which is the starting temperature of swelling, DD also showed the highest value because it had the highest amylose content (DD > SG > GH > IP > SH). Trough viscosity among different rice starches ranged from 37.13 to 141.8 cP, the lowest for DD and the highest for GH. Breakdown, setback, and final viscosity were indicated low values when the amylose content was high. Break down of DD was 29.96 cP and that of SG was 40.96, whereas the others above 100 cP. Setback value, the recovery of the viscosity during cooling of the heated starch suspension, was 51.21 cP at DD and the others showed a lower value in the order of SG < GH < SH < IP. Final viscosity value was higher than the other values may be because of the aggregation of the amylose molecules (Miles et al., 1985 a,b). Final viscosity of DD was 88.34 cP and the others above 200 cP.

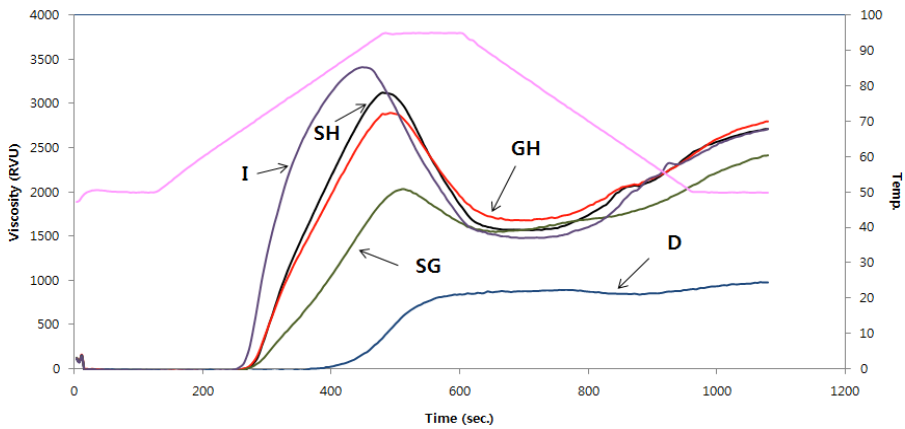


Fig. 3. RVA viscograms of rice starches.

Table 9. RVA pasting properties of rice starches.

Rice starch	Amylose content (%)	Pasting Temp. (°C)	Viscosity(cP)				
			Peak	Trough	Break down	Setback	Final
DD	66.76	91.08	73.67	37.13	29.96	51.21	88.34
IP	20.69	67.68	290.3	125.6	161.8	101.0	226.5
SG	34.00	70.88	171.8	129.5	40.96	72.38	201.9
SH	18.38	69.13	286.5	139.2	134.1	96.05	235.2
GH	21.78	69.18	249.5	141.8	103.6	91.08	232.9

4.1.1.3. Molecular weight distribution curve by HPSEC

The molecular weight distribution curves of the rice starches were shown in Fig. 4. Large peak eluted between 18-30 min represents amylopectin and that of 30-50 min represents amylose. DD with the highest content of amylose exhibited the largest peak area.

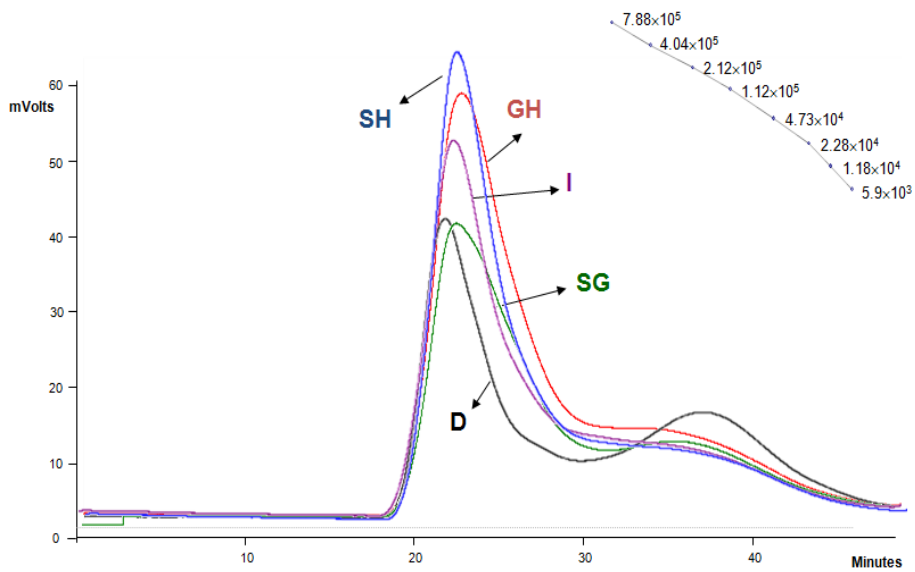


Fig. 4. The molecular weight distribution curve of rice starches.

4.1.1.4. Distribution of branched amylopectin chain length by HPAEC-PAD

HPAEC chromatograms of the chains of debranched rice starches were displayed in Fig. 5, and the distribution of branched amylopectin chain length (degree of polymerization, DP) was showed in Fig. 6 and Table 10. Amylopectin branch chains are classified by the DP range with the following chain types: A chains (DP 6-12), B1 chains (DP 13-24), B2 chains (DP 25-36), and B3+ chains (DP $\geq$ 37) (Hanashiro, 1996).

DPs of the branched chains of all starches were ranging from DP6 to DP60. In the case of SH, GH and IP were not showed difference with the distribution of the branched amylopectin chain length. It was indicated that SG has slightly higher content of short 'A' chain (34.78%) than other cultivars, but the lowest content of B3+ chain (6.81%). DD had the lowest content of 'A' chain (20.31%), while the contents of B1, B2 and B3+ chain were shown significantly higher than other cultivars. Takeda et al. (1989) reported that rice varieties with high amylose content are more distributed in the B chain with long chain length than those with low amylose content, which is consistent with the result of DD with high amylose content in this study.

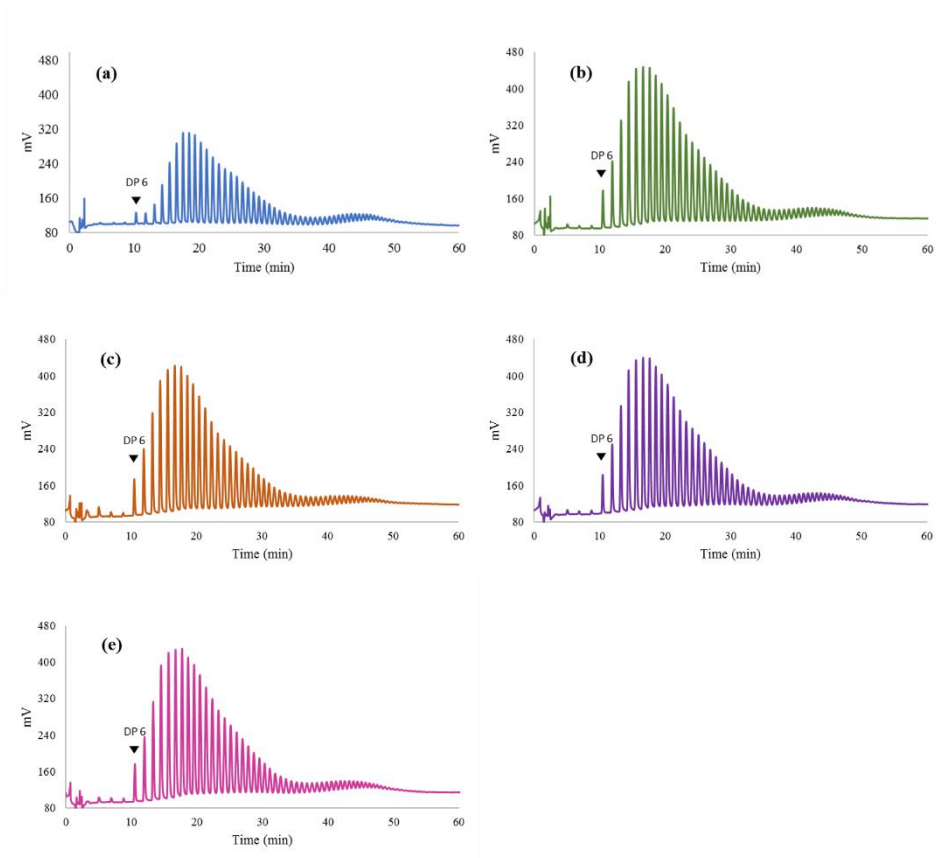


Fig. 5. HPAEC analysis of rice starches: (a) DD, (b) IP, (c) SG, (d) SH, and (e) GH.

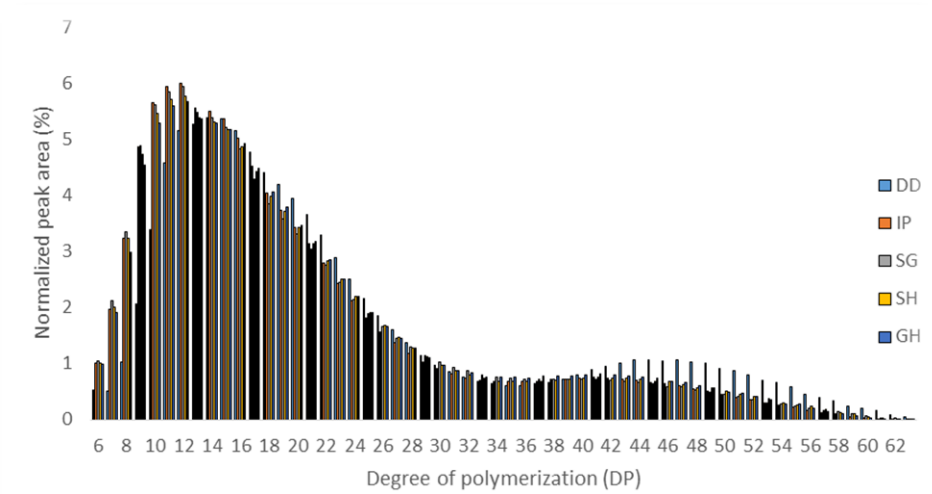


Fig. 6. Distribution of branched amylopectin chain length of rice starches.

Table 10. Distribution of degree of polymerization of rice starches.

Rice starch	Distribution (%)			
	A(DP6-12)	B1(DP13-24)	B2(DP25-36)	B3+(DP≥37)
DD	17.27	50.91	13.31	18.50
IP	28.74	47.71	12.24	11.30
SG	28.85	46.44	13.36	11.35
SH	27.96	47.09	12.91	12.04
GH	27.06	47.36	13.15	12.43

4.1.1.5. Crystallinity of granules by XRD

The X-ray spectrum of DD starch was of the 'B' type representative of tuber starches and the other four starches; IP, SG, SH, GH showed the characteristic 'A' pattern of cereal starches with three main peaks at 15.0°, 17-18° and 22-23° of 2θ (Fig. 7). Crystalline regions were formed due to sequential packing of double helices (Sarko & Wu, 1978) that were formed between the flexible 'A' chains of amylopectin (Kainuma & French, 1972), while amylose molecules formed amorphous regions in the starch structure (Cheetham & Tao, 1998). Therefore, the low X-ray intensity pattern (B type) of DD starch was probably due to its high moisture and amylose content, but low amylopectin content.

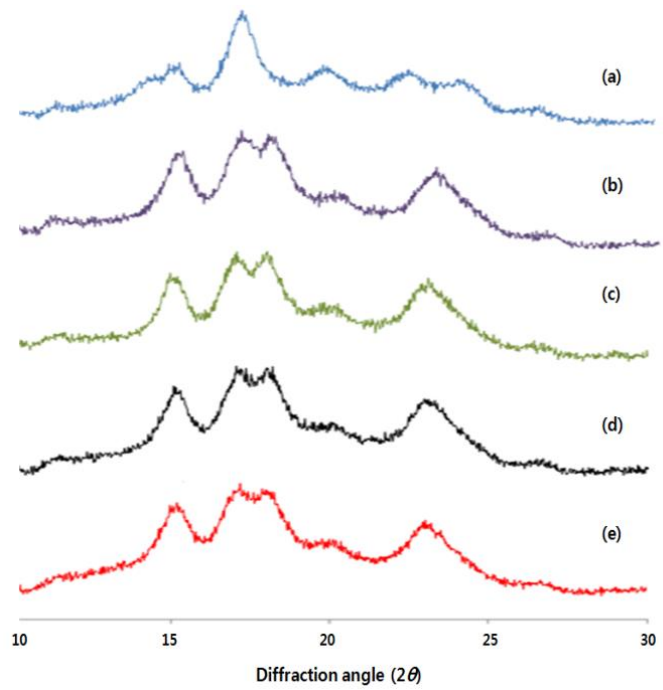


Fig. 7. X-ray diffraction patterns of rice starches: (a) DD, (b) IP, (c) SG, (d) SH, and (e) GH.

4.1.1.6. Thermal properties by DSC

Onset temperature (T_o) and peak temperature (T_p) of DD starch were significantly higher than the other starches (Table 11). Gelatinization temperature by DSC was reported to be affected by the molecular structure of amylopectin and the branched amylopectin chains that DP was less than or equal to 10. They were known to lower the gelatinization temperature because they reduced the stability of the starch crystallinity within the double helix structure. It seemed that DD showed high gelatinization temperature because 'A' chain content (DP6-12) of amylopectin and pasting temperature had a negative correlation.

The gelatinized starches were stored at 4℃ for a week and measured thermal properties of the retrograded starches. Enthalpy change of DD and SG was respectively 3.20 J/g and 1.14 J/g, which was proportional to content of amylose. The retrograded starches were hardly indicated endothermic curve on a DSC thermogram.

Table 11. Thermal properties of gelatinized and retrograded rice starches.

Rice starch		T_o(℃)	T_p(℃)	ΔH(J/g)
Gelatinization	DD	64.92 ± 0.32	73.79 ± 0.32	9.31 ± 0.16
	IP	57.07 ± 0.31	62.26 ± 0.31	9.03 ± 0.23
	SG	57.30 ± 0.07	62.32 ± 0.07	9.17 ± 0.38
	SH	57.99 ± 0.29	63.2 ± 0.29	9.05 ± 0.40
	GH	58.05 ± 0.32	63.22 ± 0.32	8.93 ± 0.60
Retrogradation	DD	40.73 ± 0.22	56.97 ± 0.75	3.20 ± 0.25
	IP	41.56 ± 0.97	48.08 ± 1.14	0.62 ± 0.09
	SG	39.65 ± 0.86	46.95 ± 0.21	1.14 ± 0.10
	SH	42.33 ± 0.39	49.76 ± 1.13	0.51 ± 0.08
	GH	41.58 ± 1.02	49.80 ± 0.92	0.67 ± 0.05

4.1.1.7. Granule morphology by SEM

The granular morphology of rice starches from different rice cultivars showed considerable variation in size and shape as viewed by SEM. SH, GH, SG and IP starches showed polygon forms and the surface of granule was smooth and angular shape. DD starch showed smooth and almost spherical shape which is different shape with other starches (Fig. 8.).

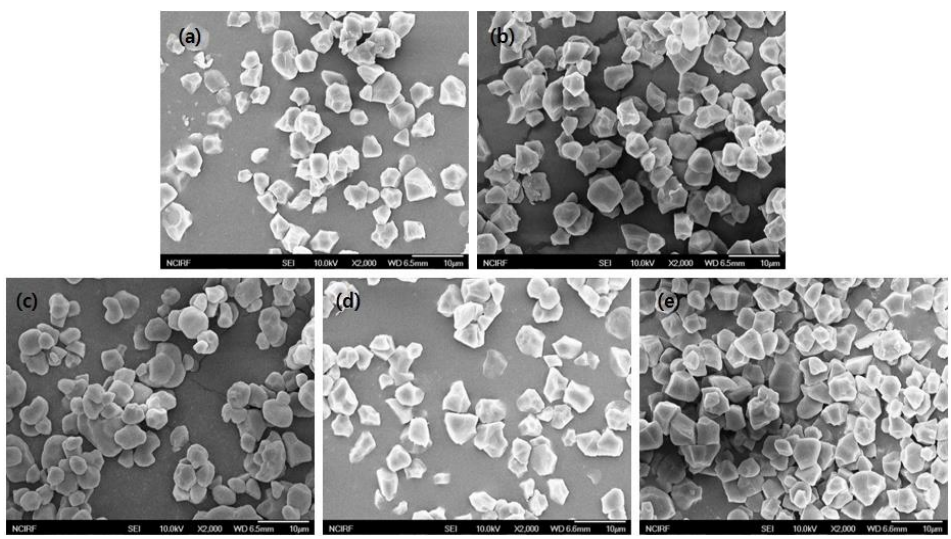


Fig. 8. Granular morphology of rice starches by SEM (× 2000): (a) SH, (b) GH, (c) DD, (d) SG, and (e) IP.

4.1.2. CD production from various rice cultivars

4.1.2.1. Reducing sugars formed from rice starch after debranching

The reducing sugars of all debranched starches (DD, IP, SG, SH, GH) were rapidly increased for initial 6 h with isoamylase treatment at 40℃ and gradually reached a plateau (Fig. 9). Therefore, the optimization of debranching time is 8 h that the highest quantity of reducing sugars formed.

The apparent amylose content was gradually increased when treatment time of isoamylase increased because linear α-1,4-glucan which can be a complex with iodine molecule was formed a lot while α-1,6-branch of starch was cut by isoamylase. Hydrolysis was continued until 8 h after isoamylase treatment, but longer treatment time decreased the apparent amylose content or reached a plateau. This is because if the enzyme treatment time is over 8 h, a little change occurs. This result was consistent with the result of reducing sugars formed after debranching (Table 12).

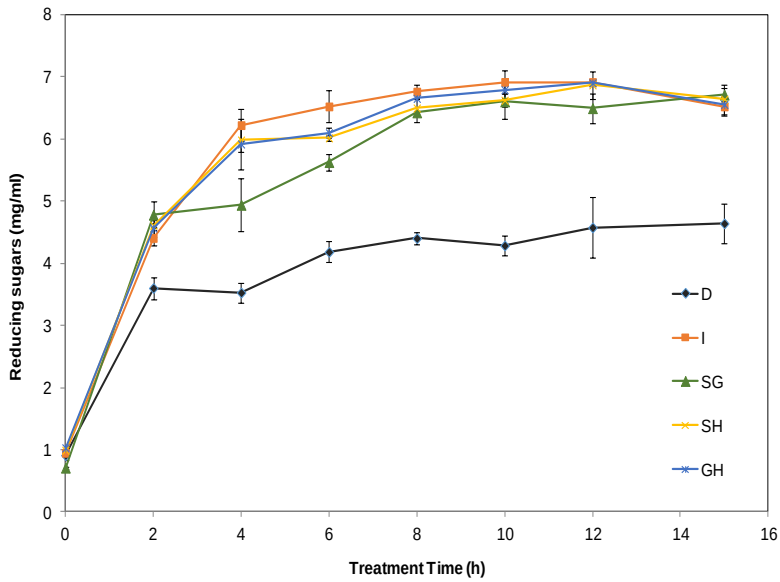


Fig. 9. Reducing sugars of debranched rice starches treated with isoamlase.

Table 12. The apparent amylose content of rice starches as the reaction time of isoamylase.

Debranching time (h)	Apparent amylose content (%)				
	DD	IP	SG	SH	GH
0	50.94±1.90	20.71±0.45	32.69±0.15	17.56±0.18	21.35±1.00
2	61.76±1.02	42.44±3.81	48.93±2.11	43.53±1.18	47.44±3.26
4	59.96±3.86	56.47±3.17	52.64±2.99	44.36±3.63	45.26±2.72
6	61.45±1.75	59.94±2.09	59.03±0.22	58.65±3.72	59.42±1.54
8	68.72±0.66	60.58±2.63	65.11±1.53	68.53±4.26	64.42±3.17
10	63.41±1.02	58.78±3.72	64.91±1.82	60.26±0.91	60.38±1.09
12	59.70±0.15	58.21±2.54	61.51±0.66	60.32±2.27	56.47±3.35
15	57.69±1.97	57.12±2.45	56.97±3.86	56.79±3.63	59.36±2.54

4.1.2.2. Optimization of CGTase and glucoamylase

In order to optimize the condition of producing CDs, debranched rice starch (1% w/v) was treated with 10 and 25U/g (at the enzyme concentration, all 'g' refers to the dry weight of the starch) of CGTase (354. 67 ± 9.70 U/ml) at 60℃ and 10 and 50 U/g of glucoamylase (170 U/ml) at 40℃ under defined time. At first, the reducing sugars of the oligosaccharide chains formed by glucoamylase were measured to know whether CD can be decomposed by glucoamylase or not (data not shown). 1% β-CD was treated with glucoamylase (5-25 U/g) to determine the degree of formation of reducing sugar up to 24 h. The reducing sugar did not increase until 24 h at all concentrations, indicating that glucoamylase from *Hormoconis resinae* cannot hydrolyze CD. This result was consistent with the report by Bender & Hans (1981) that only *Bacillus polymyxa* and a hydrolytic enzyme of *Bacillus macerans* can decompose the CDs.

After that, the degree of reducing sugar of 1% CGTase treated SG (10 and 25 U/g, 14 h) was measured using 10 and 50 U/g of glucoamylase up to 24 h (Fig. 10). SG starch with 10 U/g of CGTase had relatively low quantity of reducing sugar than the 25 U/g of CGTase treated because the higher the concentration of enzyme, the faster the coupling reaction occurred. In the case of 10 U/g of CGTase, there was no significant difference between the 10 U/g and 50 U/g of glucoamylase-treatment because there was not much oligosaccharide initially. However, in 25 U/g of CGTase, 50 U/g of glucoamylase formed more reducing sugar than 10 U/g of glucoamylase after 4 h, because the activity of the enzyme was further increased as the enzyme concentration was increased. Studies on the kinetics of enzymatic

action have shown in most cases and over certain concentration ranges that the enzymatic activity was related linearly to the concentration of enzyme and excessively to the concentration of substrate (Chance, 1943). Thus, 50 U/g of glucoamylase was considered to be more suitable than 10 U/g for increasing the purity of the CD and quantifying through the peak area, hydrolyzing oligosaccharides more than 10 U/g.

The reaction time of glucoamylase (50 U/g) was optimized by identifying the decomposition of maltodextrin (MD) and calculating peak area of CDs using HPAEC-PAD. The standard curve of CDs was obtained by commercial α-, β, and γ-CD with various concentrations (Fig. 11). After 6 h of glucoamylase treatment in the CGTase reaction mixture, most MDs were decomposed and the samples treated for 12 h showed similar results (Fig. 12). Also, it was confirmed that the peak of the CDs after 6 h were smaller than that of 0 h. This is because the oligosacchrides attached to CDs were hydrolyzed by glucoamylase. The yield of CDs at 6 h and at 12 h were 72.6 ± 4.6% and 69.0 ± 5.9%, respectively that almost same value (data not shown). As a result, 50 U/g of glucoamylase for 6 h was determined as optimum condition of obtainig the yield of CDs.

The optimum concentration of CGTase was also obtained by calculating the peak areas of CDs treated with 50 U/g of glucoamylase for 6 h using HPAEC-PAD (Fig. 13). The CD contents were rapidly increased during initial 2 h (CGTase 25 U/g) and 4h (CGTase 10 U/g) and then reached maximum at 6 h (CGTase 25 U/g) and 12 h (CGTase 10 U/g), respectively. After that time, the yield of CDs were decreased. This result might be related with the inhibition of the CGTase activity

by produced CDs during the enzyme reaction time (Moriwaki et al., 2009), and coupling reactions degrading α- or β-CDs (Uitdehaag et al., 1999).

The maximum yield of CDs from 1% starch with 25 U/g CGTase was 76.64 ± 2.81% almost same as 79.38 ± 6.51% of 10U/g, so the concentration of CGTase was determined as 25 U/g and the reaction time was 6h to reduce the producing time. This result also similar with 84.6% w/w of Duan et al. (2013) and higher than 67% from soluble starch (Yim et al., 1997) and 47% from raw corn starch (Kim et al., 1997).

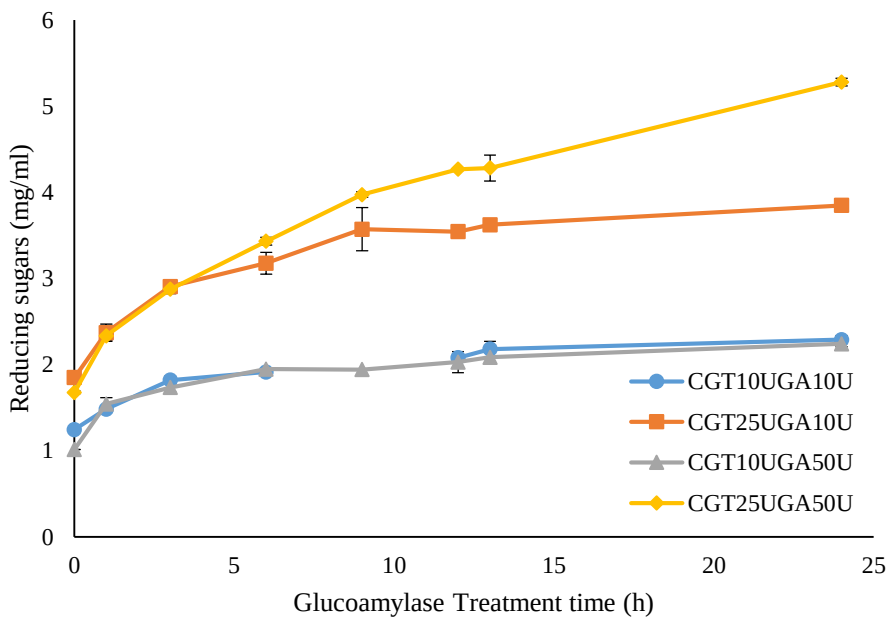


Fig. 10. Reducing sugars of CGTase (10 U/g and 25 U/g for 14 h) and glucoamylase (GA, 10 U/g and 50 U/g)-treated starches from SG measured to optimize the condition of glucoamylase.

(a)

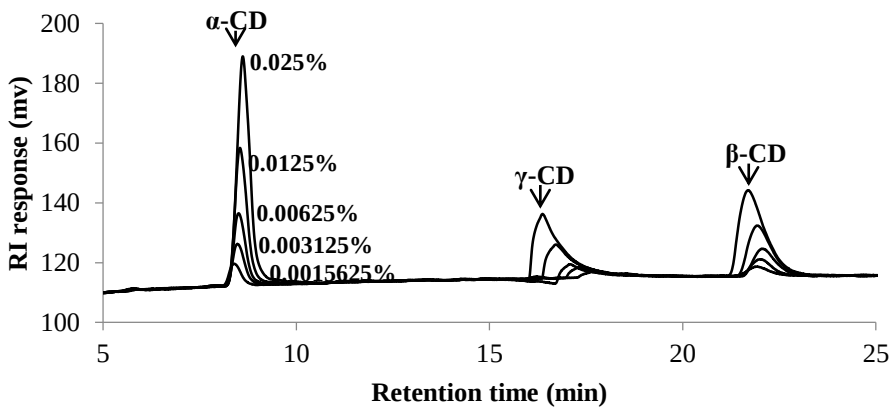
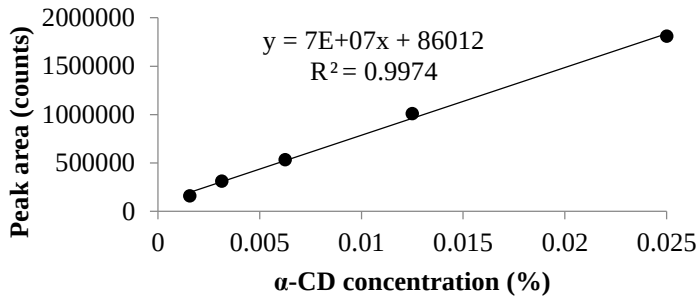
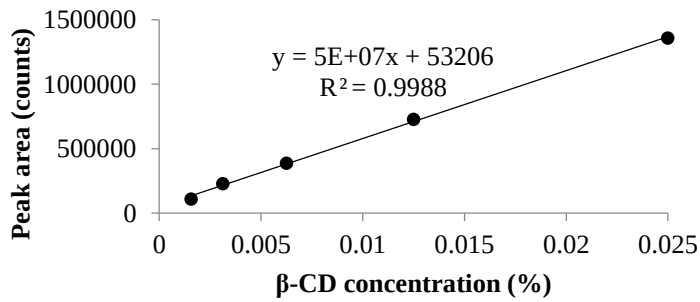


Fig. 11. (a) HPAEC analysis of the CD standard with different concentration (0.0015625-0.025% w/v) (continued on the next page).

(b)



(c)



(d)

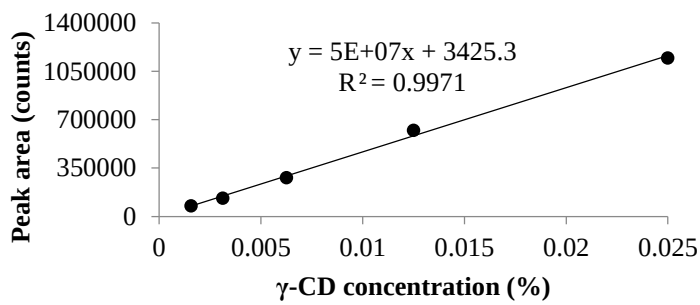
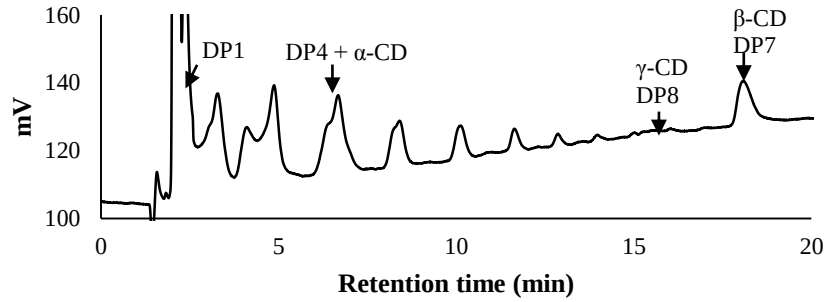
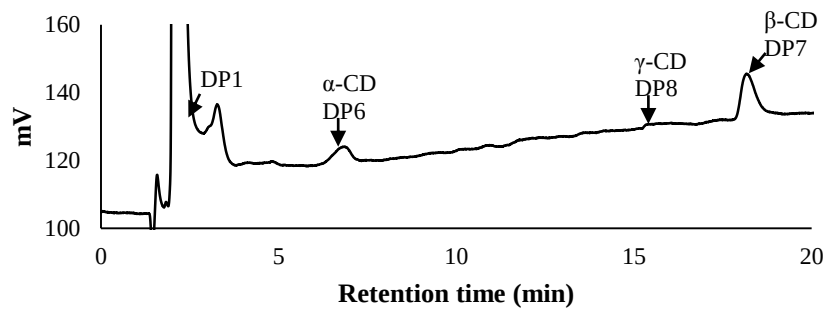


Fig. 11. (b) The standard fitting curve of α-CD based on the HPAEC results, (c) β-CD standard curve, (d) γ-CD standard curve.

(a)



(b)



(c)

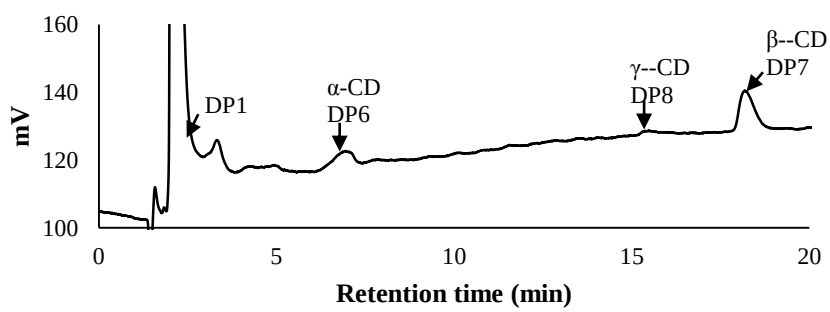


Fig. 12. HPAEC analysis for optimizing reaction time of 50 U/g glucoamylase. (a) 0 h, (b) 6 h, and (c) 12 h.

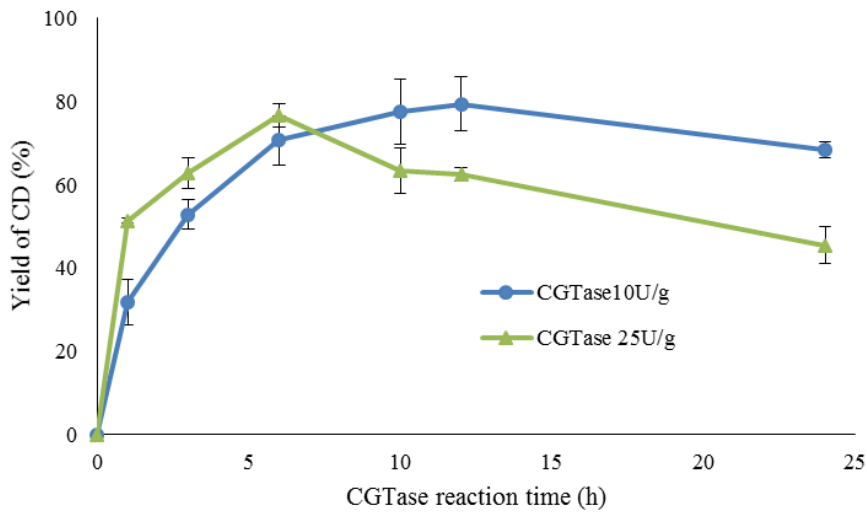


Fig. 13. Time course analysis of CD production yield by different CGTase concentration (10 and 25 U/g).

4.1.2.3. Comparison of CD yield from various rice cultivars

CDs were produced under optimum conditions of enzymes (5 U/g of isoamylase for 8 h at 40℃, pH 4.5; 25 U/g of CGTase for 6 h at 60℃, pH6.0; 50 U/g of glucoamylase for 6 h at 40℃, pH 4.5) from various rice cultivars: DD, IP, SG, SH, and GH. The conversion yields of CD calculated by peak area using HPAEC were appeared in Fig. 14. There was no statistical difference between five starches, but DD showed the highest yield of CD (82.91% w/w) and GH produced the lowest yield of CD (70.16% w/w) (Table 13).

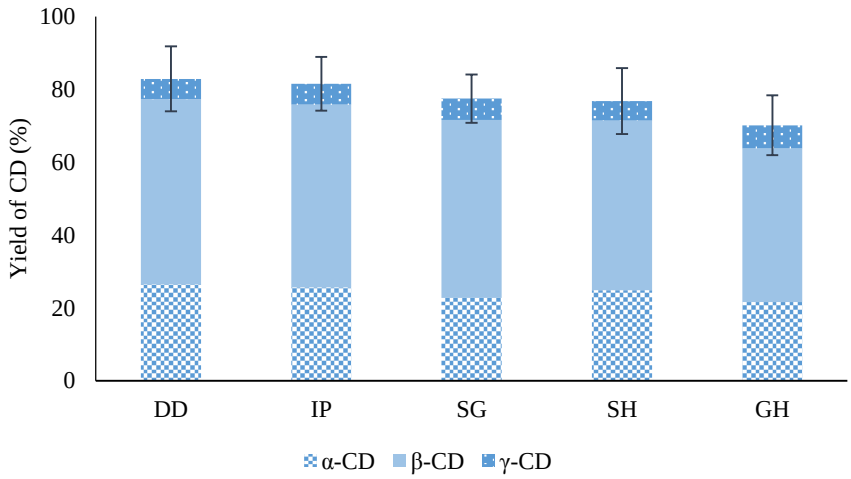


Fig. 14. Yield of CD from DD, IP, SG, SH, and GH.

Table 13. Yield of CD from DD, IP, SG, SH, and GH.

starch	Yield of CD (%)			
	α-CD	β-CD	γ-CD	total-CD
DD	26.52	50.84	5.55	82.91 ± 8.94
IP	25.64	50.24	5.65	81.53 ± 7.38
SG	22.77	48.86	5.86	77.49 ± 6.66
SH	24.85	46.59	5.40	76.84 ± 9.07
GH	21.66	42.16	6.35	70.16 ± 8.21

4.1.2.4. Identification of CD products by MALDI-TOF MS

In order to prove that the CD product was cyclic structure, their molecular masses were measured by MALDI-TOF MS (Fig. 15). The molecular masses of cyclic- and noncyclic-glucans were calculated as 162.14n + 22.990 Da (mass of Na+ ion) and 162.14n + 22.990 + 18.015 Da (mass of reducing end residue) respectively, where n is the number of monosacchride (glucose) unit. It was indicated that the CD products was purely cyclic structure with DP 6, 7, and 8. Among them, the intensity of β-CD (Mw of $[M+Na]^+$=1157 Da) was the greatest and the γ-CD intensity was the lowest (Mw of $[M+Na]^+$=1319 Da).

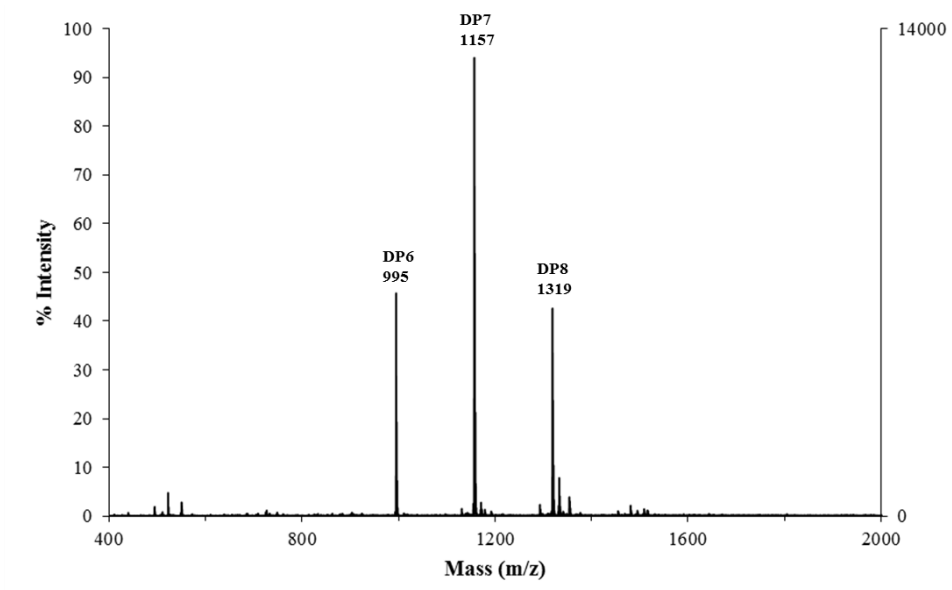


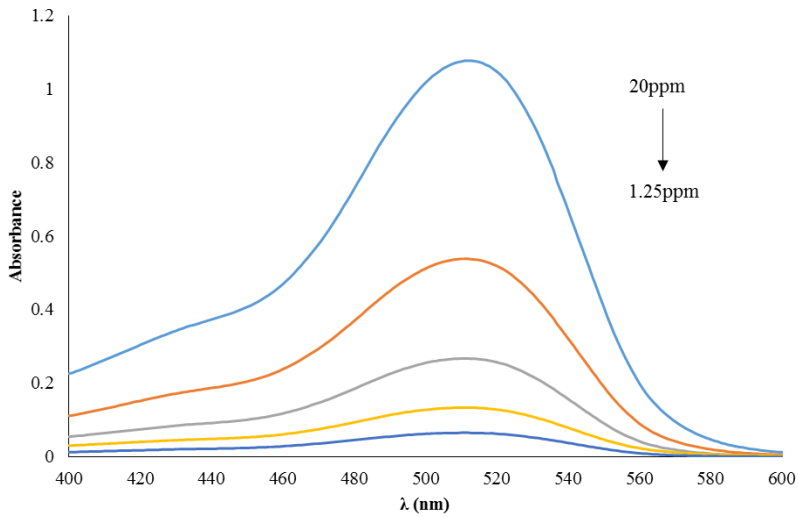
Fig. 15. MALDI-TOF analysis for CD produced by 25 U/g CGTase. The number above each peak showed the DP and the molecular mass (in daltons) of the molecule plus 23 Da (Na+).

4.2. Interaction between anthocyanin and CD produced from high amylose rice starch

4.2.1. Extraction of ACN from black rice

ACN was extracted with 60% (v/v) ethanol and 0.2% (v/v) hydrogen chloride from SH (section 3.3.1). In order to examine the concentration of extracted ACN, UV-Vis absorption spectra of commercial ACN were recorded at pH 2 on-line from 400 to 600 nm using UV-Vis spectrophotometer. The standard curve was prepared on the basis of the ACN absorbance of each ACN concentration (1.25 – 20 ppm) at 511 nm, $Y = 0.054X - 0.0023$, $R^2 = 1$ where Y was assigned for absorbance of ACN and X was assigned for the concentration of ACN (Fig. 16). The absorbance of extracted ACN was measured at 511 nm and the concentration was calculated by fitting it in a standard curve. As a result, the concentration of ACN extracted solution was 320.05 ± 14.74 ppm.

(a)



(b)

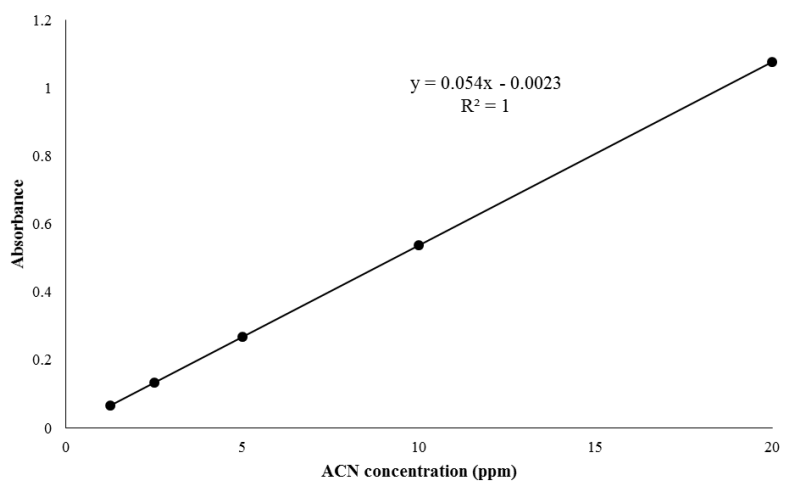


Fig. 16. (a) UV-Vis absorption spectra of commercial ACNs at pH 2. (b) The standard fitting curve of ACNs based on the UV-Vis absorption at 511 nm.

4.2.2. Interaction of ACN with CD

4.2.2.1. Production of CD

The solubility of commercial β-CD was 1.8% (w/v), so maximum concentration of β-CD was 2% (w/v) when thermal- and photo-stability tests were conducted. In order to compare the effect of β-CD and produced DD-CD, higher concentration of CD than 0.83% (w/v) from 1% (w/v) debranched DD starch was needed. Thus, the substrate concentration was increased to 2, 3, and 5% (w/v) and DMSO, which may interfere with ACN complex formation, was removed. When 25 U/g of CGTase treated to each debranched DD starches at 60℃, the CD concentration at 4 h of reaction time from 2% (w/v) DD starch was 0.36% and 3% starch produced 0.61% (w/v) of CDs. 5% (w/v) DD starch produced 0.88% (w/v), the highest CD concentration of all (data not shown). Therefore, the substrate concentration for the mass production of CD was determined to be 5% (w/v). In order to identify the optimum concentration of CGTase and reaction time, the concentration of CGTase tested from 5 U/g to 50 U/g for 24 h at 60℃ (Fig. 17). The maximum concentration of CD (1.24 % w/v) was obtained by 5 U/g of CGTase-treatment for 4 h. The concentration of CD was quantified by calculating peak area using HPAEC (section 3.2.3.5.) after 0.5 U/g of glucoamylase was treated for 6 h. The maximum concentration of DD-CD starch with 5 U/g of CGTase for 4 h was 1.58% (w/v) (Fig. 18), which is almost same as the solubility of β-CD: 1.8% (w/v). The calculated proportion of starch bioconversion to CD obtained was 32.64% (w/w), lower than other reported cases. Szerman et al. (2007) obtained 66% (w/w) of converted CD from 5% (w/v) cassava starch with CGTase from *Bacillus circulans* DF 9R and Yim et al. (1997) produced 48% yield of CD from 5% soluble starch.

The lower yield might be caused from the cooling of the CGTase-treated samples (stored at 4℃) after centrifugation, followed by filtration with 0.45 μm pore sized filter for removing long-chain polymers. As the solubility of the dissolved main β-CDs was reduced by the low temperature compared to immediately after CGTase-treatment, the CDs were remained only as much as the solubility of β-CD (1.8% w/v) in the reaction mixture after removing the aggregated CDs by filtering. Trends in Fig. 17 seemed to be different from those in Fig. 13. Reaction conditions for Fig. 13 and 17 were different especially for the substrate condition and the existence of DMSO, which might produce those differences.

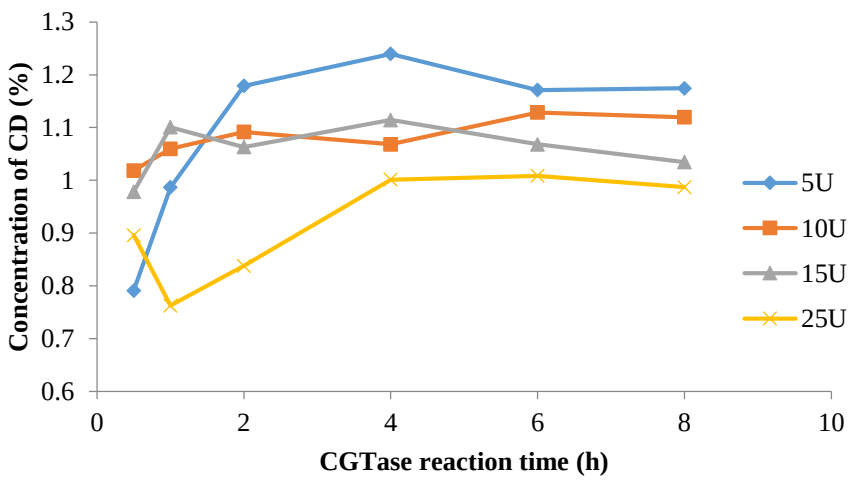


Fig. 17. Optimization of concentration (5-25 U/g) and reaction time (for 8 h) of CGTase by calculating peak area of CDs using HPAEC.

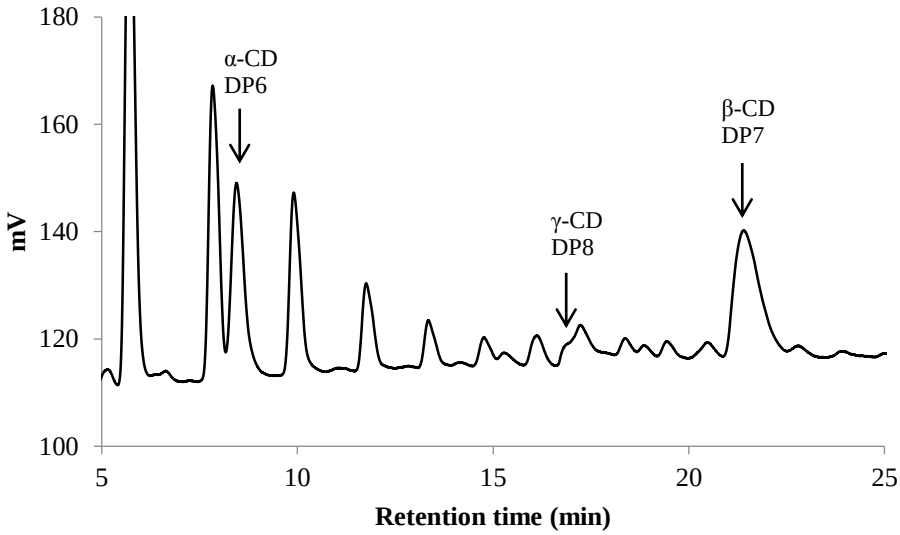


Fig. 18. HPAEC anaylsis of produced CD from 5% DD starch with optimum condition (5 U/g of CGTase treatment for 4 h). α-, β-, and γ-CD were indicated above each peak with the DP.

4.2.2.2. Fading effect of ACN with CD

The decrease of the absorbance of ACN according to the change of β-CD concentration and pH was observed by UV-Vis spectrophotometer from 280 to 680 nm (Fig. 19). The results showed that the maximum absorbance of ACN decreased by increasing pH level. This is due to the phenomenon that ACN is reddish color with flavylium cation as main structure at pH 2, and decolorized into hemiketal structure which gradually becomes less color as pH increases (Fig. 19a). This variation in absorbance of ACN was decreased as the concentration of β-CD increased (Fig. 19b&c), because the ACN is complexed with β-CD, leading to shift of the equilibrium to the hemiketal which is a stable structure at a high pH (anti-copigmentation effect) (Fernandes et al., 2013). Fig. 20 indicated that the change of absorbance at 511 nm over pH and β-CD concentration. As the concentration of β-CD and the pH of ACN increased, the degree of decline in absorbance at 511 nm tended to decrease gradually. This suggests that the absorbance of ACN was relatively low due to the increase of β-CD participating in complex formation with ACN at high concentration of β-CD and the high variation of absorbance at pH 2 seemed to be due to the increase of anti-copigmentation effect.

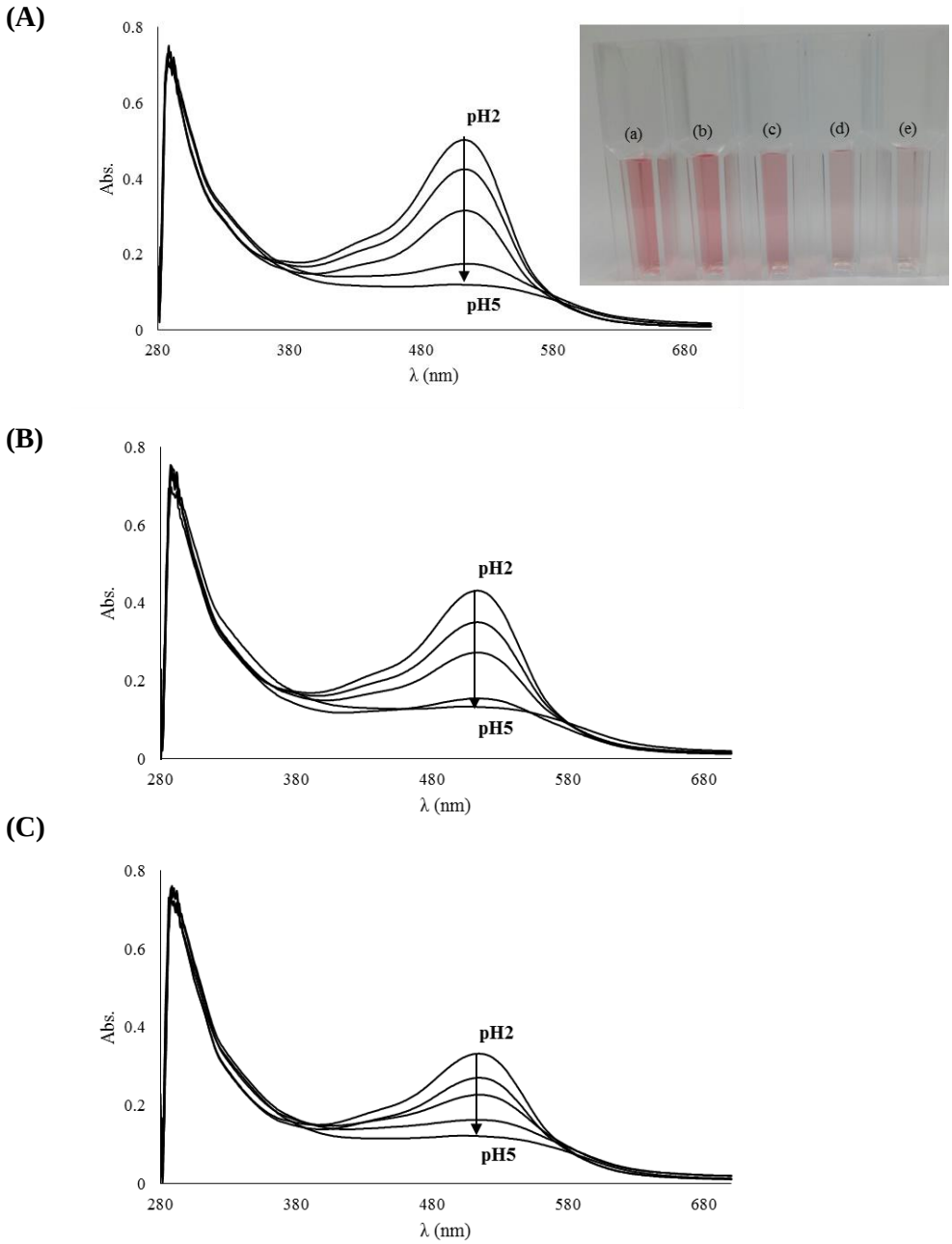


Fig. 19. UV-Vis spectra and color of ACN as various pH level (pH 2-5) at each concentration of β-CD at 1:0-20 ratio: (A) 1:0, (B) 1:5, (C) 1:20. At (A), (a) pH 2, (b) pH 2.5, (c) pH 3, (d) pH 4, and (e) pH 5.

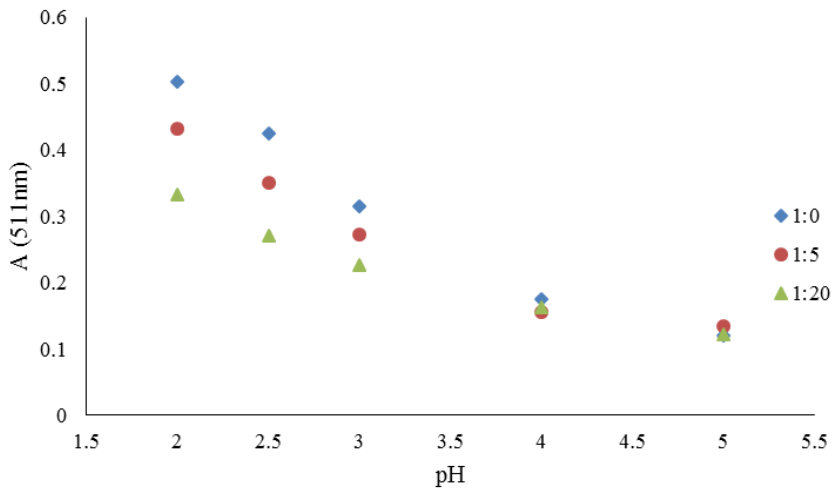


Fig. 20. Fitting absorbance value of ACN with various concentration of β-CD and pH level using UV-Vis spectrophotometer at 511 nm.

4.2.3. Analysis of stability of ACN with CD

4.2.3.1. Antioxidant activity as various concentration of ACN with CD

To determine the concentration range of ACN that can be measured by the ABTS method, differences in antioxidant activity at ACN concentrations of 0.001, 0.01, and 0.1% were compared at pH 4 (Fig. 21). As a result, antioxidant activity increased to 0.34%, 3.82%, and 31.28%, respectively, as the concentration of ACN increased from 0.001% to 0.1%. The antioxidant activity was not significantly affected by increasing the amount of β-CD added (0 to 2% w/v) at all ACN concentrations. Therefore, the concentration of ACN was determined to be 0.1%, which is the most suitable antioxidant activity to exhibit the antioxidant activity within the pH range of 2-8 based on the value at pH 4 (31.28%).

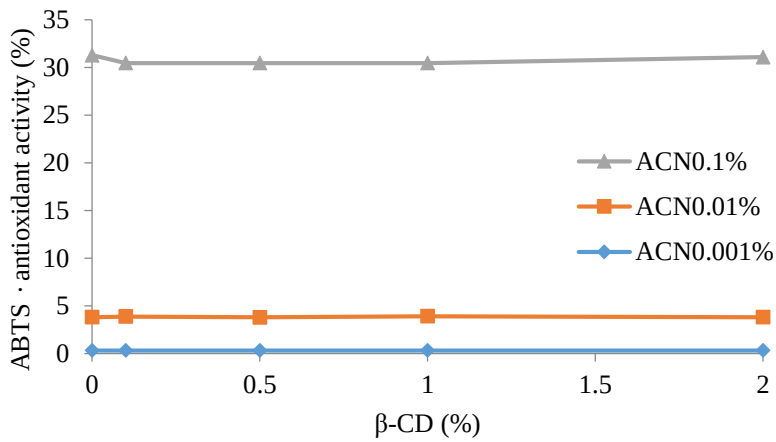


Fig. 21. Change in antioxidant activity with ACN concentration by addition of β-CD using ABTS assay.

4.2.3.2. Antioxidant activity as various pH level of ACN with CD

The antioxidant activity of ACN on β-CD concentrations (0 to 2% w/v) was compared at pH 2, 4, 6 and 8 (Fig. 22). As a result, the antioxidant capacity of anthocyanin showed an increasing tendency with increasing pH level, which was similar to that of the previous study (Borkowski et al., 2005). There was no statistically significant difference in the antioxidant capacity by β-CD concentration at low pH, but there was a slight difference in antioxidant capacity due to the increase in pH. As the concentration of β-CD increased at pH 8, the antioxidant activity tended to increase.

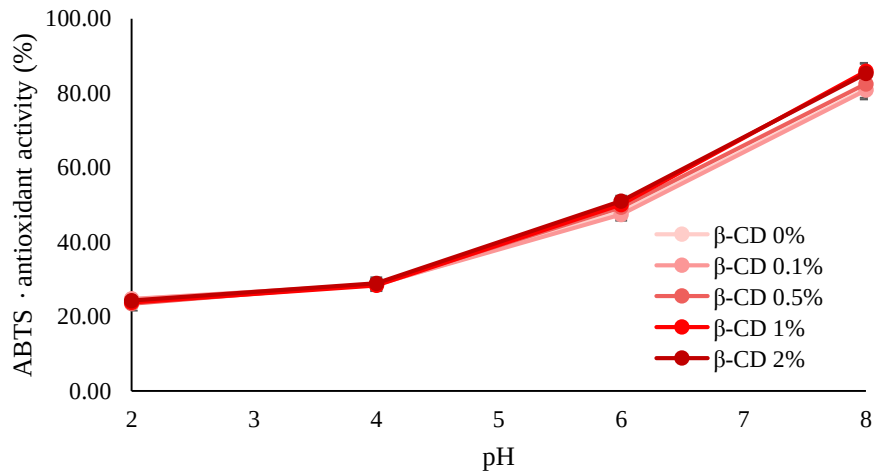


Fig. 22. Antioxidant activity of ACN by β-CD (0, 0.1, 0.5, 1, 2% w/v) according to pH (2, 4, 6, 8).

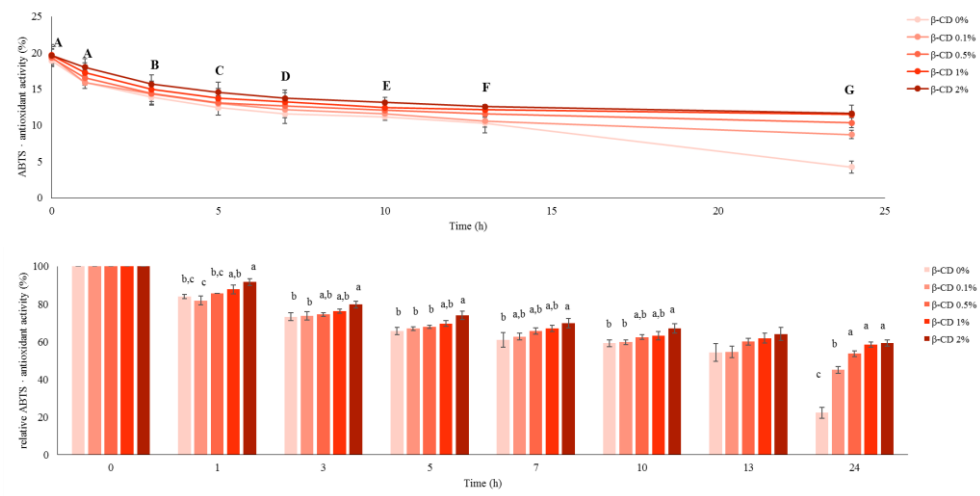
4.2.3.3. Thermal stability

The stability of ACN antioxidant activity to heat was monitored for 24 h at pH 2, 4, 6, and 8 (Fig. 23). Antioxidant activity was significantly decreased with the heating time at all pH levels. There was no significant change in antioxidant activity according to β-CD concentration at the same heating time, but after 24 h of heating at 96℃, the rate of decrease in antioxidant activity was low as the β-CD concentration increased at all pH levels. In particular, at pH 2, the antioxidant activity was maintained by β-CD not only at 24 h but also at all other time points (Fig. 23a). After heating for 1 h, the antioxidant activity was about 1.1 times higher at 2% than when the concentration of β-CD was 0%, and it was similar to that of 10 h. After heating for 13 h, the value was about 1.2 times higher than 0% β-CD. After 24 h of heating, the difference was maximized, so ACN with 2% of β-CD showed about 2.7 times higher antioxidant ability than ACN without β-CD. As a result, when ACN was exposed to heat for a long time (24 h), the more antioxidative stability of ACN could be maintained as the more β-CD was added.

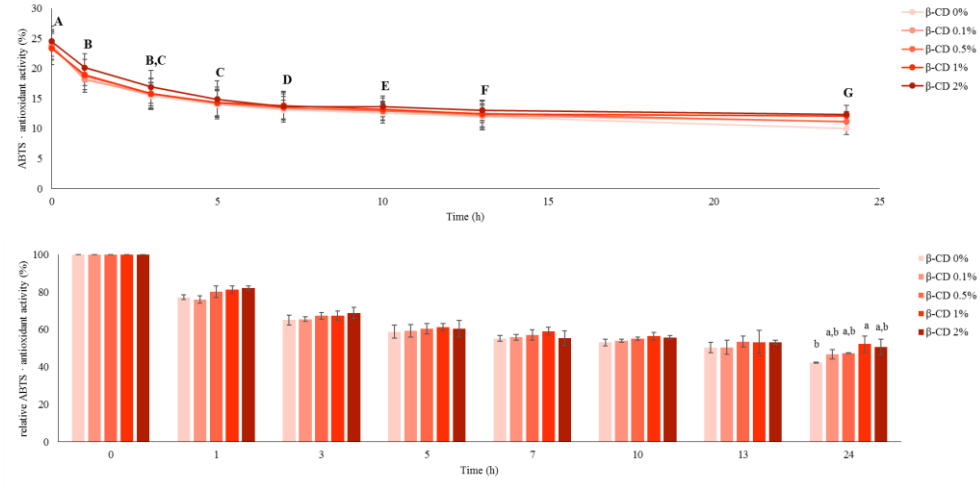
The half-life ($t_{1/2}$) of antioxidant activity of the ACN in the heat treatment was the shortest at pH 2 in the order of pH 2 <8 <6 <4, indicating that ACN was destroyed faster at pH 2 than the other pH (Table 14). However, when the concentration of β-CD was 2%, the $t_{1/2}$ of antioxidant activity was short in the order of pH 8 <6 <4 <2. The degradation rate constants (k_d) were the lowest at pH 4 (2.69×10^{-2}/h) in order of pH 4 <6 <8 <2, which was the opposite of $t_{1/2}$. In 2% β-CD, the values were low in order of pH 2 <4 <6 <8. This result suggests that ACN exists in a relatively stable structure at pH 4 and weak structure at pH 2 under heat treatment conditions.

The effect of DD-CD and MD on thermal stability of ACN was also investigated in the same method as in section β-CD. Until 9h, both DD-CD and MD showed slight protective effect against destruction of photo-stability of ACN. However, after 24 h, the ACNs containing DD-CD and MD all turned brown, so it indicated a rapid increase in antioxidant activity (data not shown) and this result was reported by Tonon et al (2010). The degradation of ACN was accelerated with high temperature or long time of storage due to sugars, together with proteins, which can result in the Maillard reaction (non-enzymatic browning). DD-CD and MD had a lot of reducing sugars unlike pure β-CD, so they made ACN colored brown after 24 h with thermal treatment. Sugars or degradation-resulting products can hasten the degradation of ACN, because the degradation rate follows the conversion rate of sugars to furfural. Furfural, a derivative of aldopentoses, as well as hydroxymethylfurfural, a derivative of keto-hexoses, are products of the Maillard reaction condensed with ACNs and form brown colored compounds (Von Elbe & Schwartz, 1996).

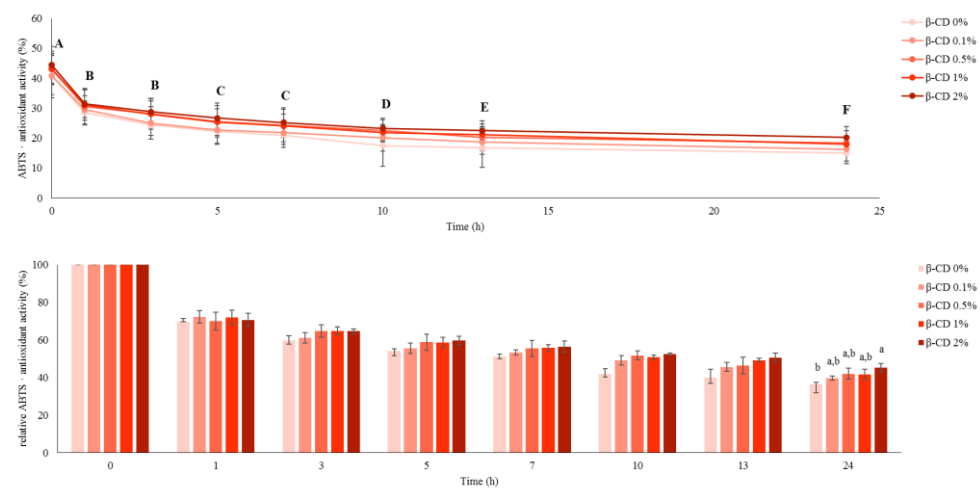
(a)


ABTS - antioxidant activity (%)
Time (h)
β-CD 0%
β-CD 0.1%
β-CD 0.5%
β-CD 1%
β-CD 2%
relative ABTS - antioxidant activity (%)
Time (h)

(b)


ABTS - antioxidant activity (%)
Time (h)
β-CD 0%
β-CD 0.1%
β-CD 0.5%
β-CD 1%
β-CD 2%
relative ABTS - antioxidant activity (%)
Time (h)

(c)



(d)

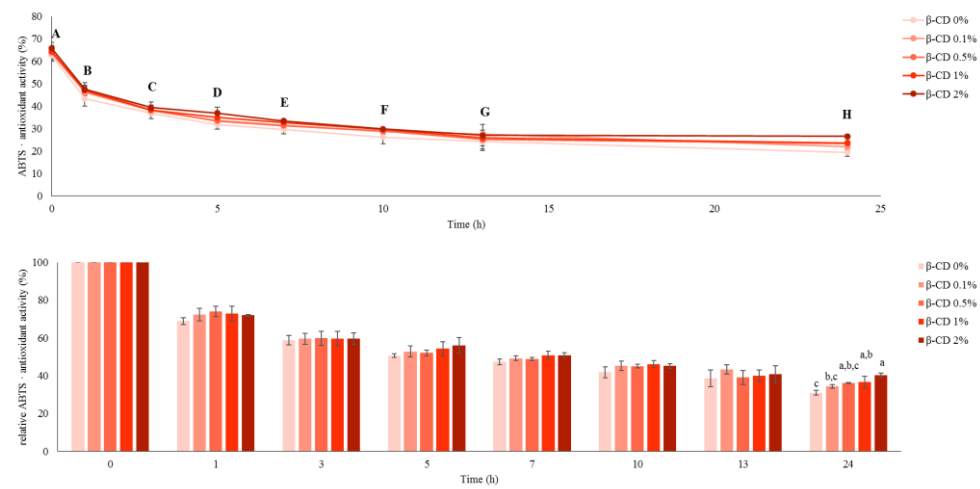


Fig. 23. Thermal stability of ACN antioxidant activity by β-CD concentration (0-2% w/v) for 24 h with heat treatment (96℃): (a) at pH 2, (b) at pH 4, (c) at pH 6, (d) at pH 8.

Table 14. (a) Decomposition rate constant (k_d) and (b) half-life ($t_{1/2}$) by β-CD concentration and pH for 24 h with heat treatment (96℃).

(a)

$k_d(10^{-2}/h)$	pH2	pH4	pH6	pH8
β-CD_0%	5.58±0.57[a]	2.96±0.01[NS1)]	3.63±0.70[NS]	4.15±0.28[NS]
β-CD_0.1%	2.95±0.21[b]	2.57±0.09	3.14±0.20	3.70±0.04
β-CD_0.5%	2.26±0.06[b]	2.57±0.02	2.94±0.18	3.65±0.21
β-CD_1%	2.00±0.09[b]	2.26±0.45	2.95±0.32	3.59±0.57
β-CD_2%	2.01±0.22[b]	2.10±0.35	2.58±0.17	3.21±0.25

1) No significant difference

(b)

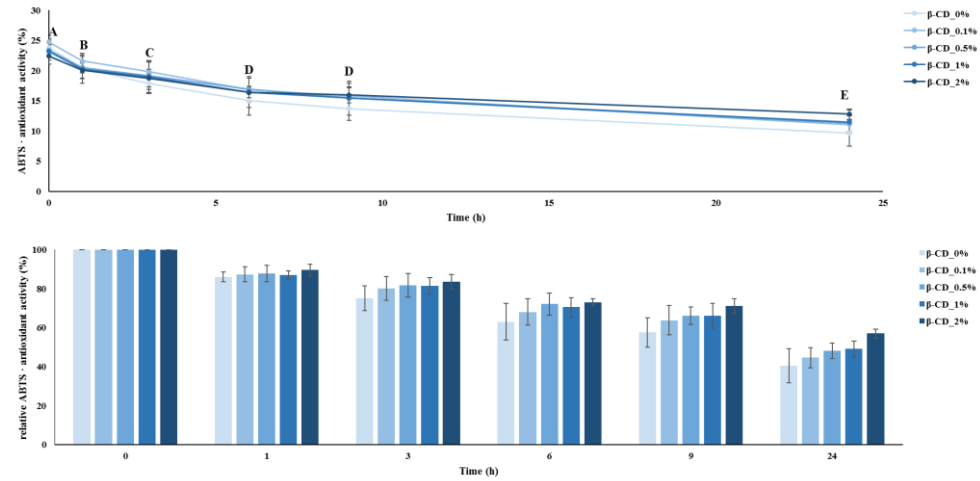
$t_{1/2}$(h)	pH2	pH4	pH6	pH8
β-CD_0%	12.54±1.20	23.39±0.10	19.75±3.33	16.79±1.10
β-CD_0.1%	23.83±1.78	27.00±0.94	22.16±1.42	18.75±0.19
β-CD_0.5%	30.68±0.78	27.00±0.26	23.68±1.37	19.05±1.08
β-CD_1%	35.38±0.93	32.17±7.51	23.79±2.72	19.76±3.02
β-CD_2%	34.94±3.76	29.62±4.84	27.01±1.80	21.70±1.78

4.2.3.4. Photo stability

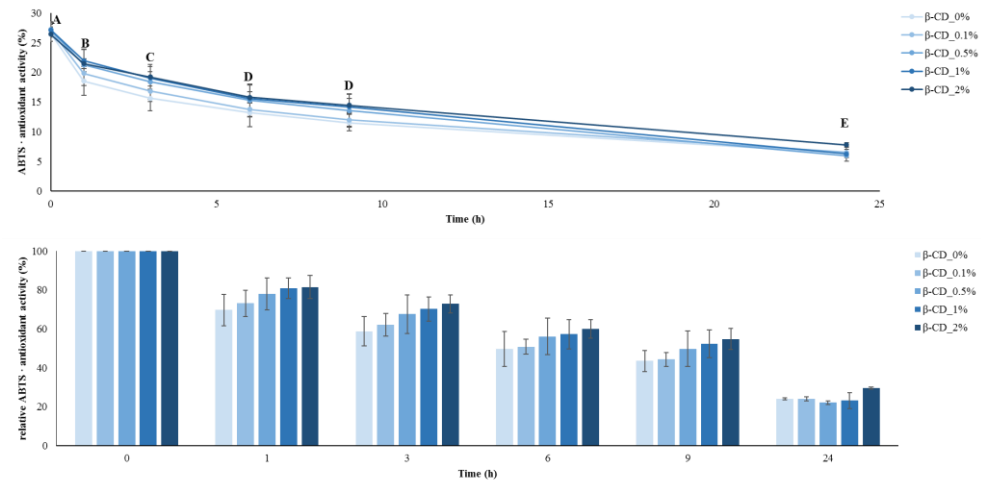
Photo (UVB) stability of complexed ACN with β-CD at pH 2, 4, 6, and 8 was monitored in UVB chamber for 24 h with 0-2% w/v concentration of β-CD (Fig. 24). Antioxidant activity of ACN decreased with increasing UVB irradiation time at all pH levels. At pH 2, the antioxidant activity of 2% concentration of β-CD tended to decrease about 1.4 times less than ACN without β-CD, indicating that photo stability was increased (Fig. 24a). At pH 4 and 6, the addition of β-CD showed change in antioxidant activity until 9 hours, but no significant difference after 24 h of UVB irradiation. As a result, when the ACN was exposed to UVB, the antioxidative stability of ACN was maintained as the addition of β-CD at pH 2 was observed.

The $t_{1/2}$ values of ACN antioxidant activity with UVB irradiation time was the shortest at pH 4 in the order of pH 4 <6 <2 <8 (Table 15). However, when the concentration of β-CD was 2%, the $t_{1/2}$ of antioxidant activity was short in the order of pH 4 <6 <8 <2. The degradation rate constants (k_d) were the lowest at pH 8 (2.29×10^{-2}/h) in order of pH 8 <2 <6 <4, which was the opposite of $t_{1/2}$. In 2% β-CD, the values were low in order of pH 2 <8 <6 <4. This result suggests that ACN exists in a relatively stable structure at pH 8 and weak structure at pH 4 under UVB irradiation conditions.

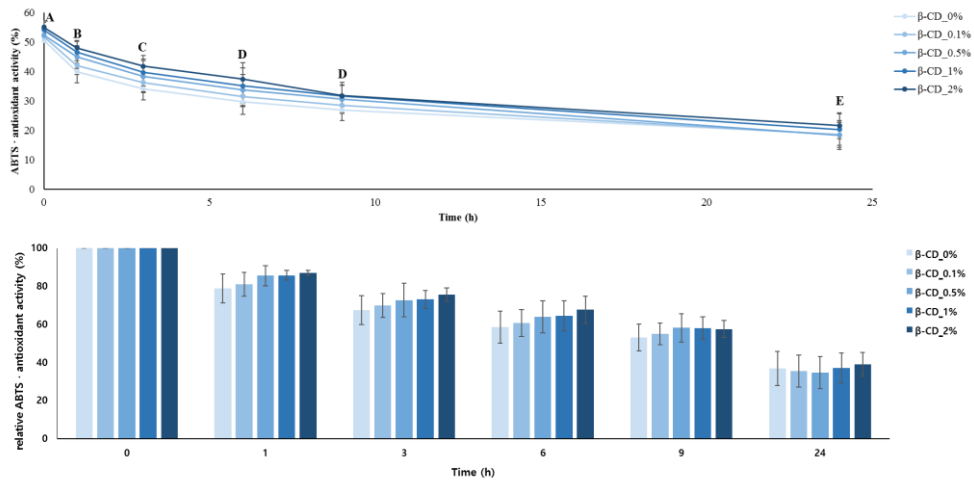
(a)


ABTS - antioxidant activity (%)
Time (h)
A
B
C
D
D
E
β-CD_0%
β-CD_0.1%
β-CD_0.5%
β-CD_1%
β-CD_2%
relative ABTS - antioxidant activity (%)
Time (h)

(b)


ABTS - antioxidant activity (%)
Time (h)
A
B
C
D
D
E
β-CD_0%
β-CD_0.1%
β-CD_0.5%
β-CD_1%
β-CD_2%
relative ABTS - antioxidant activity (%)
Time (h)

(c)



(d)

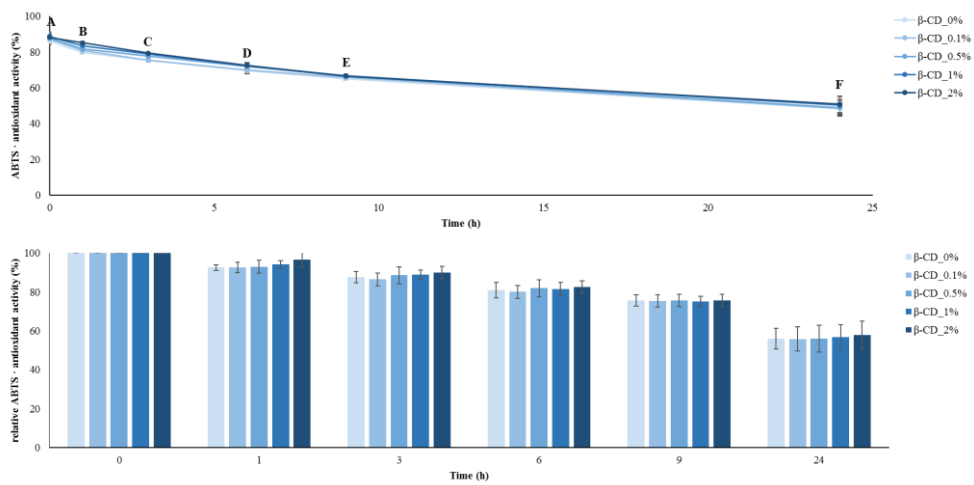


Fig. 24. Photo stability of ACN antioxidant activity by β-CD concentration (0-2% w/v) for 24 h with UVB: (a) at pH 2, (b) at pH 4, (c) at pH 6, (d) at pH 8.

Table 15. (a) Decomposition rate constant (k_d) and (b) half-life ($t_{1/2}$) by β-CD concentration and pH for 24 h with UVB irradiation.

(a)

$k_d(10^{-2}/h)$	pH2	pH4	pH6	pH8
β-CD_0%	3.52±0.75[a]	5.12±0.21[NS1)]	3.71±0.72[NS]	2.29±0.34[NS]
β-CD_0.1%	3.14±0.38[a,b]	5.24±0.34	3.93±0.76	2.30±0.38
β-CD_0.5%	2.79±0.20[a,b]	5.78±0.13	4.14±0.77	2.33±0.41
β-CD_1%	2.70±0.30[a,b]	5.68±0.87	3.86±0.75	2.30±0.41
β-CD_2%	2.10±0.11[b]	4.66±0.21	3.69±0.59	2.27±0.43

1) No significant difference

(b)

$t_{1/2}$(h)	pH2	pH4	pH6	pH8
β-CD_0%	20.78±5.16	13.55±0.54	19.55±4.40	30.94±4.97
β-CD_0.1%	22.45±2.97	13.28±0.86	18.43±4.11	31.05±5.78
β-CD_0.5%	24.95±1.90	12.00±0.28	17.41±3.71	30.80±6.08
β-CD_1%	26.00±2.73	12.48±1.86	18.78±4.18	31.30±6.36
β-CD_2%	33.14±1.68	14.89±0.65	19.33±3.46	31.75±6.77

4.2.3.5. Photo-stability of ACN with produced CD and MD

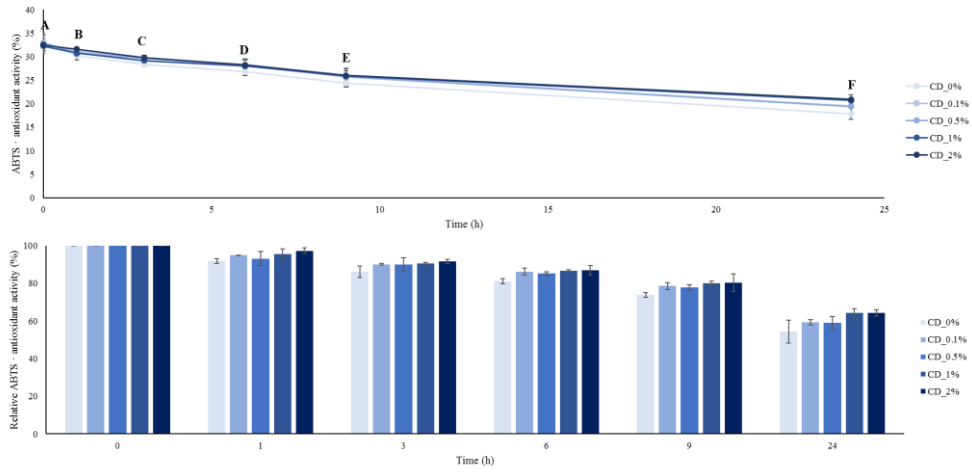
The effect of CD produced from DD and MD on photo-stability of ACN was investigated in the same method as in section 4.2.3.4 (Fig. 25). Although there were no statistically significant differences, the results showed that 2% of DD-CD had a stabilizing effect on ACN more than same concentration of MD. As can be seen from Fig. 25, the antioxidant activity of free ACN was below 60%, whereas that of its complexation with 2% of DD-CD was 64.33% and 2% of MD was 61.38% slightly lower than DD-CD at pH2 after 24h (Fig. 25a). The difference of antioxidant activity between free ACN and complexed with DD-CD was maximized at pH4 (Fig. 25b). After 24h, the antioxidant activity of ACN complexed with 2% of DD-CD was 1.16 times higher than that of free ACN, whereas that of MD was slightly lower than that of free ACN.

The $t_{1/2}$ of ACN antioxidant activity with UVB irradiation time was shorter at pH 4 than pH2 (Table 16). When the concentration of DD-CD was 2%, the $t_{1/2}$ of antioxidant activity was 8.92 h longer than that of free ACN and 2% MD indicated 2.87 h longer than that of free ACN at pH2. At pH 4, the $t_{1/2}$ of antioxidant activity of ACN with 2% DD-CD was 3.97 h longer than free ACN, but that of ACN with 2% MD showed 0.29 h shorter than that of free ACN. The k_d values of ACN was the opposite of the $t_{1/2}$ values. They were lower at pH 2 than at pH 4, and decreased as the concentration of DD-CD and MD was increased except that of MD at pH 4 (Table 16a).

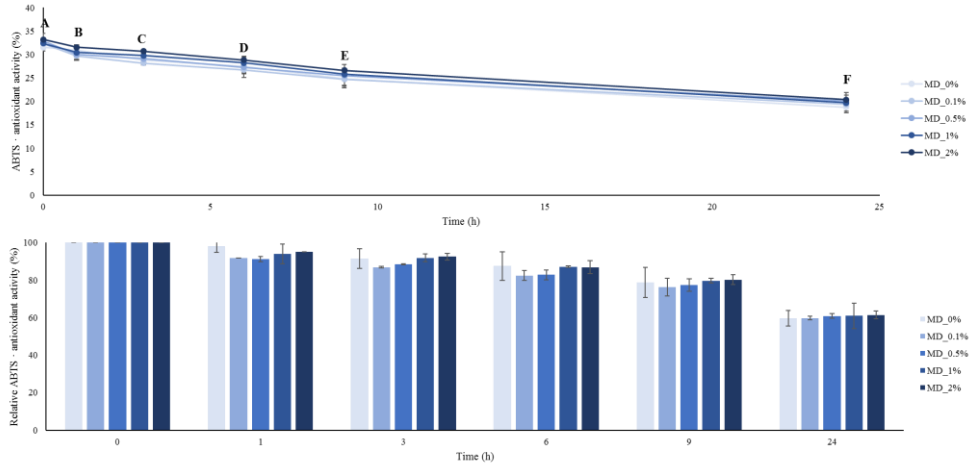
The effects of pure β-CD, DD-CD, and MD on the photo-stability of ACN were compared in Table 17. The difference value of k_d between free ACN and complex

ACN was showed that 2% β-CD was the most effective molecule enhancing the photo-stability of ACN at pH 2. However, 0.1-1% of DD-CD was superior to that of β-CD and MD (Table 17b). Also, the difference both k_d and $t_{1/2}$ values of DD-CD was the highest at pH 4 from the overall concentration range. Therefore, DD-CD is most effective in improving the photo-stability of free ACN in the overall concentration interval: DD-CD > β-CD >MD.

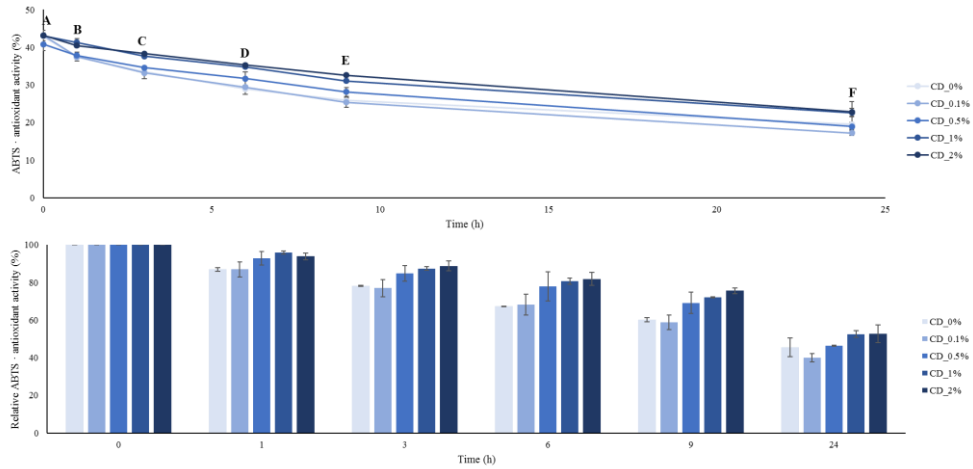
(a)


ABTS · antioxidant activity (%)
Time (h)
A
B
C
D
E
F
CD_0%
CD_0.1%
CD_0.5%
CD_1%
CD_2%
Relative ABTS · antioxidant activity (%)
Time (h)

(b)


ABTS · antioxidant activity (%)
Time (h)
A
B
C
D
E
F
MD_0%
MD_0.1%
MD_0.5%
MD_1%
MD_2%
Relative ABTS · antioxidant activity (%)
Time (h)

(c)



(d)

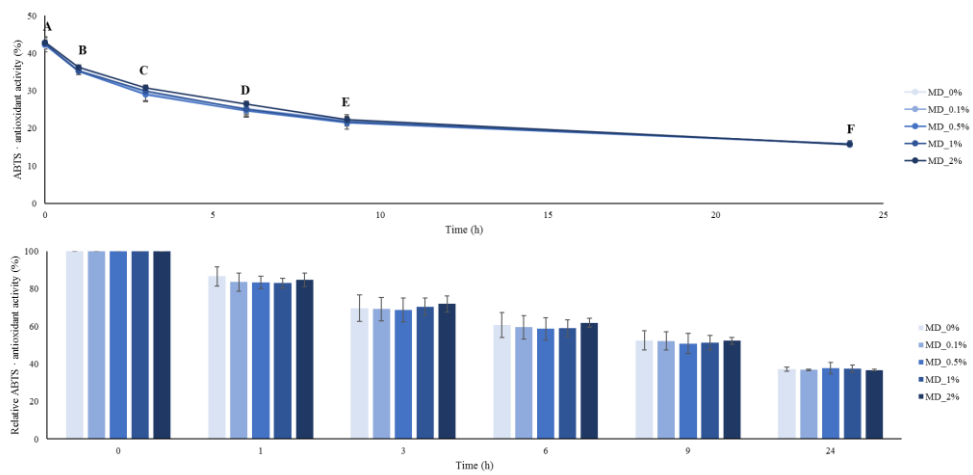


Fig. 25. Photo stability of ACN antioxidant activity by DD-CD and MD concentration (0-2% w/v) for 24 h with UVB: (a) DD-CD effect at pH 2, (b) MD effect at pH 2, (c) DD-CD effect at pH 4, (d) MD effect at pH 4.

Table 16. (a) Decomposition rate constant (k_d) and (b) half-life ($t_{1/2}$) by DD-CD and MD concentration and pH for 24 h with UVB irradiation.

(a)

$k_d(10^{-2}/h)$	pH2		pH4	
	DD-CD	MD	DD-CD	MD
0%	2.42±0.42	2.16±0.20	3.03±0.50	3.76±0.70
0.1%	2.10±0.11	1.98±0.08	3.55±0.12	3.73±0.23
0.5%	2.04±0.18	1.89±0.07	3.03±0.02	3.77±0.27
1%	1.78±0.10	2.02±0.37	2.63±0.16	3.69±0.11
2%	1.81±0.13	1.98±0.16	2.60±0.44	3.82±0.07

(b)

$t_{1/2}$(h)	pH2		pH4	
	DD-CD	MD	DD-CD	MD
0%	29.57±5.08	32.36±3.00	23.54±3.85	18.44±0.34
0.1%	33.16±1.66	35.06±1.42	19.57±0.63	18.67±1.13
0.5%	34.24±3.02	36.81±1.27	22.91±0.11	18.50±1.30
1%	39.15±2.10	35.56±6.44	26.50±1.56	18.80±0.56
2%	38.49±2.76	35.23±2.85	27.51±4.72	18.15±0.33

Table 17. The difference values of k_d (a) and $t_{1/2}$ (b) between free ACN and complexed ACN with β-CD, DD-CD, and MD at pH 2 and pH4 for 24 h with UVB irradiation.

(a)

$k_d(10^{-2}/h)$	pH2			pH4		
	β-CD	DD-CD	MD	β-CD	DD-CD	MD
0%	3.52±0.75[a]	2.42±0.42	2.16±0.20	5.12±0.21	3.03±0.50	3.76±0.70
0.1%	3.14±0.38[a,b]	2.10±0.11	1.98±0.08	5.24±0.34	3.55±0.12	3.73±0.23
0.5%	2.79±0.20[a,b]	2.04±0.18	1.89±0.07	5.78±0.13	3.03±0.02	3.77±0.27
1%	2.70±0.30[a,b]	1.78±0.10	2.02±0.37	5.68±0.87	2.63±0.16	3.69±0.11
2%	2.10±0.11[b]	1.81±0.13	1.98±0.16	4.66±0.21	2.60±0.44	3.82±0.07

(b)

$t_{1/2}$(h)	pH2			pH4		
	β-CD	DD-CD	MD	β-CD	DD-CD	MD
0%	20.78±5.16	29.57±5.08	32.36±3.00	13.55±0.54	23.54±3.85	18.44±0.34
0.1%	22.45±2.97	33.16±1.66	35.06±1.42	13.28±0.86	19.57±0.63	18.67±1.13
0.5%	24.95±1.90	34.24±3.02	36.81±1.27	12.00±0.28	22.91±0.11	18.50±1.30
1%	26.00±2.73	39.15±2.10	35.56±6.44	12.48±1.86	26.49±1.56	18.80±0.56
2%	33.14±1.68	38.48±2.76	35.23±2.85	14.89±0.65	27.51±4.72	18.15±0.33

Ⅵ. Summary and conclusions

Starches from five different Korean special rice cultivars, Dodammi (DD), Ilpummi (IP), Saegoami (SG), Sintoheukmi (SH), and Geonganghongmi (GH) were isolated and analyzed their physicochemical properties using RVA, HPSEC, HPAEC, XRD, DSC, and SEM. The results showed that DD had the highest amylose content among five starches. The maximum yield of CDs produced by treatment of CGTase and glucoamylase was 82.91% from 1% of debranched DD starch, which might be attributed to high amylose content that converted into linear glucan by isoamylase and allowing the enzyme reaction to start from a great number of points. The yields of CDs were obtained by calculating peak area using HPAEC, and confirmed by MALDI-TOF MS.

The pH dependence and CD effect of anthocyanin (ACN) extracted from SH was confirmed with UV-Vis spectra and antioxidant activity by ABTS assay. The structure of ACN was changed depending on pH, so fading effect from red color at pH 2 to colorless at pH 4 was indicated with increased β-CD concentration. Antioxidant activity was increased as pH increased, but not as β-CD concentration increased except at pH 8. Thermal and photo-stability of ACN complexed with 0-2% concentration of β-CD, CD from 5% of debranched DD starch (DD-CD), and maltodextrin (MD) were investigated, respectively. The thermal stability of ACN was more improved by β-CD, compared to that of free ACN and the photo-stability of that was more improved by β-CD and DD-CD than that of free ACN and MD. The reducing sugars of DD-CD and MD caused Miallard reaction of ACN at high temperature, so the further studies of thermal stability of ACN complexed with

DD-CD and MD will be needed in a different way. In addition, ACN is easily degradated by various conditions, so studies on stability under conditions other than heat and light are also necessary.

In conclusion, CD production from Korean special rice starches with debranching enzyme and extraction of ACN from SH were tried for the first time. Also, the stabilizing effect of CD was pH dependent, especially at pH 2 and 4. In the alkaline range, no effect of CD was observed. The CGTase-reaction mixture containing CD from rice starch showed similar or better performance with commercial β-CD probably due to other stabilizing component such as α-, γ- CD, and MD. Therefore, the development of antioxidant-strengthening materials with improved stability is expected to be feasible.

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국문초록

흑미는 다양한 유용 성분, 특히 대표적인 천연 항산화제로 널리 알려져 있는 안토시아닌 (ACN) 을 포함하는 식품 작물이다. 그러나 ACN 은 천연물 중에서 가장 불안정한 화합물에 속하고 그 결정화 또한 곤란하기 때문에 이를 포함한 흑미의 응용범위가 제한되는 문제가 있다. 따라서 본 연구에서는 ACN 의 빛과 열에 대한 안정성을 증진시키기 위해 다양한 게스트 분자와 복합체를 형성 할 수 있는 다목적 호스트 분자인 환형덱스트린 (CD)를 이용하여 ACN 의 안정성의 향상을 시도하였다. 또한 다양한 쌀 전분에 isoamylase 와 *Thermoanaerobacter sp.* 유래 CGTase, glucoamylase 를 처리하여 CD 생산의 최적화를 도모하였다.

도담미 (DD), 일품미 (IP), 새고아미 (SG), 신토흑미 (SH), 건강홍미 (GH) 다섯 가지 종류의 특수미 전분의 물리화학적 특성을 분석하고 CD 의 생산 수율을 비교하였다. DD 는 66 %의 가장 높은 아밀로스 함량으로 인해 HPAEC, RVA, DSC, XRD, SEM 과 같은 다양한 물리화학적 분석 결과에서 다른 종류들과 상당한 차이를 나타내었다. CD 생산을 위한 최적 조건을 결정하기 위한 예비실험에서 SG 전분 1%에 isoamylase 5U/g 을 8 시간, CGTase 25U/g 을 6 시간, glucoamylase 50U/g 을 6 시간 처리하였을 때 최대 수율 76.64%를 보였다. 다섯 가지 전분에서 최적 조건으로 CD 를 생산하였을 때, DD 가

82.91%로 가장 높은 수율을 나타냈으며 이에 따라, DD 를 CD 생산의 최종 기질로 선택하였다.

ACN 은 추출 후, 동결 건조를 통해 분말화하였으며 CD 가 ACN 의 안정성에 미치는 영향을 조사하기 위해 0~2%의 β-CD 를 0.1% ACN 과 pH 2, 4, 6, 8 에서 24h 동안 혼합한 뒤, 열처리(96℃)와 UVB 조건에서 24h 동안 항산화능이 감소되는 정도를 ABTS 방법으로 비교 분석하였다. 열처리 조건에서는 pH 2 에서 β-CD 를 첨가할수록 ACN 의 항산화 안정성이 유지되는 유의적 차이를 나타내었고, UVB 조건에서 역시 pH 2 에서 β-CD 에 의해 항산화 안정성이 유지되는 경향을 나타냈다. pH 와 β-CD 농도 별 항산화능의 degradation constant(k_d)값과 반감기를 통해 ACN 이 pH2 에서 열에 취약한 구조로 존재하고, pH 4 에서는 UVB 에 취약한 구조로 존재하며 CD 가 이 pH 에서 ACN 을 보호 한다는 것을 유추하였다. 동일한 조건으로 pH2 와 4 에서 DD 로부터 생산된 CD(DD-CD), 말토덱스트린(MD) 각각을 첨가하여 ACN 의 항산화 안정성을 비교한 결과, 전반적으로 MD 와 기존 β-CD 의 첨가보다 DD_CD 의 첨가가 항산화 안정성에 미치는 효과가 더 높았는데, 이는 MD 보다 CD 가 ACN 과의 복합체 형성능이 뛰어나고 DD-CD 안에 들어 있는 α-, β-, γ- CD 가 단일 β-CD 에 비해 ACN 과의 복합체 형성에 높은 효과가 있다는 점을 시사한다.

결론적으로, 본 연구는 isoamylase 와 CGTase 를 순차적으로 처리하여 쌀 전분으로부터 고효율 CD 를 제조할 수 있음을 확인하였으며, CD 가 ACN 과 복합체를 형성하여 ACN 의 pH 의존성 변색과 열 및 UV 불안정성을 완화한다는 것을 확인하여 산업적 활용 가능성을 제시하였다. 그러나 DD-CD 제조시, CGTase 의 활성이 당과 CD 에 의해 저해되므로 공정 과정 중에 CD 를 분리흡착하는 과정이 요구된다. 또한, ACN 은 빛이나 열 이외의 환경에서도 불안정한 것으로 알려진 바, ACN 의 저장 안정성 및 산화 안정성에서 DD-CD 가 미치는 영향을 추가적으로 실시하여 추후 DD-CD 가 첨가된 안정한 항산화 소재의 개발과 흑미의 활용성 확대가 가능 할 것으로 사료된다.

주요어: 흑미, 안토시아닌, 환형덱스트린, 아이소아밀레이즈, 싸이클로덱스트린 글루카노트렌스퍼레이즈, 글루코아밀레이즈, 열안정성, 광분해안정성