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MINI-REVIEW

Implications of agar and agarase in industrial applications of sustainable marine biomass

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Abstract

Agar, a major component of the cell wall of red algae, is an interesting heteropolysaccharide containing an unusual sugar, 3,6-anhydro-L-galactose. It is widely used as a valuable material in various industrial and experimental applications due to its characteristic gelling and stabilizing properties. Agar-derived oligosaccharides or mono-sugars produced by various agarases have become a promising subject for research owing to their unique biological activities, including anti-obesity, anti-diabetic, immunomodulatory, anti-tumor, antioxidant, skin-whitening, skin-moisturizing, anti-fatigue, and anti-cariogenic activities. Agar is also considered as an alternative sustainable source of biomass for chemical feedstock and biofuel production to substitute for the fossil resource. In this review, we summarize various biochemically characterized agarases, which are useful for industrial applications, such as neoagarooligosaccharide or agarooligosaccharide production and saccharification of agar. Additionally, we succinctly discuss various recent studies that have been conducted to investigate the versatile biological activities of agar-derived saccharides and biofuel production from agar biomass. This review provides a basic framework for understanding the importance of agarases and agar-derived saccharides with broad applications in pharmaceutical, cosmetic, food, and bioenergy industries.

Keywords Red algae · Agar · Agarose · Neoagarooligosaccharide · Agarooligosaccharide · Biological activity · Biofuel

Introduction

Agar is the main cell wall component of several red algae, such as *Gelidium* and *Gracilaria* (Duckworth and Yaphe 1972), and it is composed of a mixture of two polysaccharides, agarose and porphyran (Araki 1959; Knutsen et al. 1994). Agarose is a linear polysaccharide that consists of an agarobiose repetitive unit (G-AHG) of β-D-galactose (G) and 3,6-anhydro-α-L-galactose (AHG), whereas porphyran is a linear polysaccharide that is composed of a porphyrobiose repetitive unit (G-L6S) of G and α-L-galactose-6-sulfate (L6S). In agar, G and AHG (or L6S) residues are linked by β-1,4 linkages, and AHG (or L6S) and G residues are linked by α-1,3 linkages (Usov 1998). Agarose and porphyran are mainly composed of agarobiose and porphyrobiose units, respectively, but they are heteropolysaccharides; consequently, even pure agarose contains small amounts of porphyrobiose, and pure porphyran contains small amounts of agarobiose (Hehemann et al. 2010).

Agarases are glycoside hydrolases (GH) that catalyze the hydrolysis of agarose. They are classified into two groups, α-agarases and β-agarases, based on their mode of action. α-Agarases hydrolyze α-glycosidic linkages of agarose, resulting in the production of agarooligosaccharides (AOs) with AHG at their reducing end, whereas β-agarases hydrolyze β-glycosidic linkages of agarose, which consequently produces neoagarooligosaccharides (NAOs) with G at their reducing end (Chi et al. 2012; Yun et al. 2017) (Fig. 1). α-Glycosidic linkages can be also cleaved randomly by acid treatment (Chi et al. 2012). α-Neoagarooligosaccharide hydrolases (α-NAOSH), when acting exogenously on the α-1,3 linkage can release AHG from NAOs. Particularly, neoagarobiose (NA2) is degraded into AHG and G by α-NAOSH (Asghar et al. 2018) or α-neoagarobiose hydrolases (α-NABH) (Day and Yaphe 1975). G can be utilized by a general transport system and catabolic pathway found in many bacteria (Frey 1996), but degradation of AHG requires two specific enzymes, 3,6-anhydro-α-L-galactose dehydrogenase and 3,6-anhydrogalactonate cycloisomerase, which have been only identified in agar-degrading bacteria (Yun et al. 2015) (Fig. 1).

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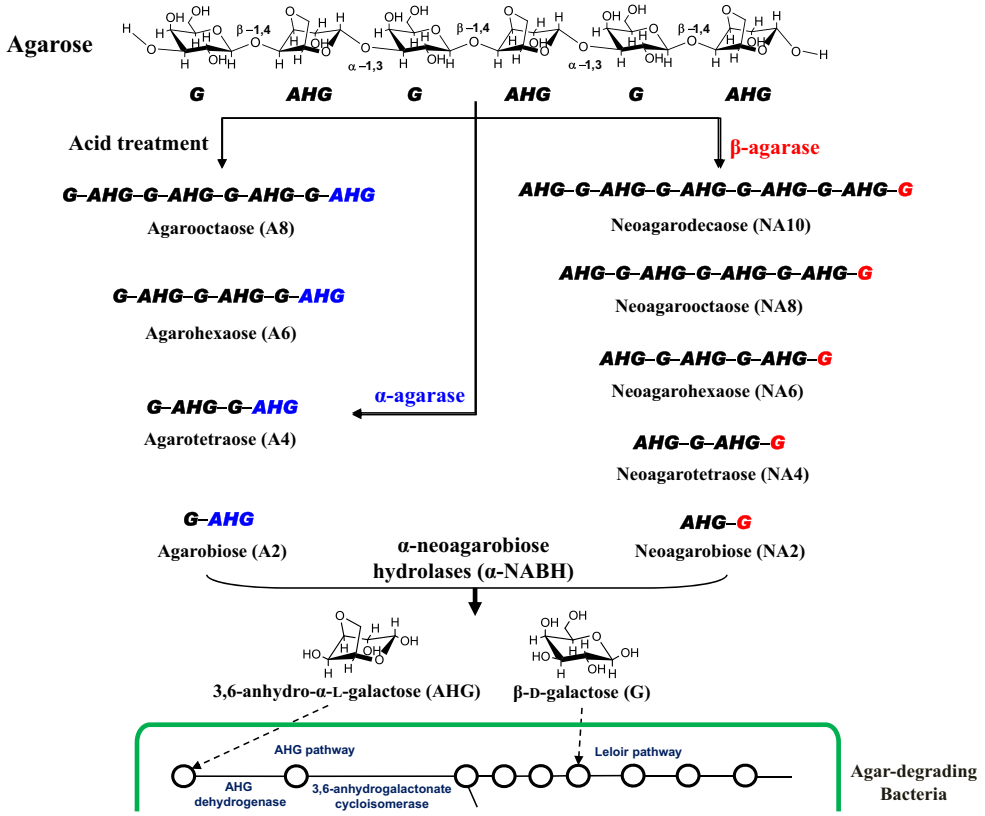

Fig. 1 Schematic diagrams of agar hydrolysis by agarases. α-Agarases or acid treatment cleave the α-1,3 linkages of agarose between 3,6-anhydro-α-L-galactose (AHG) and D-galactose (G) residues, which produces agarooligosaccharides (AOs) with AHG at their reducing end, such as agarobiose (A2), agarotetraose (A4), and agarohexaose (A6). β-Agarases cleave the β-1,4 glycosidic linkages of agarose between G and AHG residues, which produces neoagarooligosaccharides (NAOs) with G residues at their reducing ends, such as neoagarobiose (NA2), neoagarotetraose (NA4), and neoagarohexaose (NA6). NA2 is cleaved into monomeric sugars, AHG and G, by α-neoagarobiose hydrolase (NABH) or α-neoagarooligosacchride hydrolase (α-NAOSH). G can be utilized by a general transport system and catabolic pathway (Leloir pathway) found in many bacteria, but degradation of AHG requires two specific cytosolic enzymes, 3,6-anhydro-α-L-galactose dehydrogenase and 3,6-anhydrogalactonate cycloisomerase, which are identified only in agar-degrading bacteria

Therefore, agar-degrading bacteria with these enzymes can utilize AHG as the sole carbon source (Yun et al. 2015). Metabolomic analysis showed that 3,6-anhydrogalactonate, the metabolic intermediate of the AHG degradation pathway, was detected during growth only in the presence of AHG, indicating that two steps in AHG catabolism are not involved in the catabolism of other common sugars (Yun et al. 2018). A recent study demonstrated that the agarolytic β-galactosidase is also involved in agar degradation. Neoagarotetraose (NA4) transported into cells was degraded into AHG and agarotriose (A3) by intracellular α-NAOSH, and then the agarolytic β-galactosidase degraded A3 into G and NA2 (Yu et al. 2020). These results suggest that NA4 can be degraded via two different agarolytic pathways.

Owing to various and unidentified biological functions of degradation products of agarose, such as NAOs, AOs, and AHG, the search for agarases with suitable industrial applications is an important task. In this review, we will summarize the biochemical features of various agarases with unusual enzymatic properties, suitable for industrial applications. Moreover, we succinctly discuss the recent studies that have been conducted to assess the physiological activities of agar-derived sugars, as well as recent progresses in the development of efficient methods for biofuel production from agar.

Agar-degrading enzymes with unique characteristic features

β-Agarases

β-Agarases have been found in diverse bacteria isolated from various environments, including marine, soil, and plant roots, and many of them have been biochemically characterized (Chi et al. 2012; Yun et al. 2017; Jahromi and Barzkar 2018; Veerakumar and Manian 2018). β-agarases belong to various glycoside hydrolase (GH) families, including GH16, GH39, GH42, GH50, GH86, and GH118, but most β-agarases belong to two GH families, GH16 and GH50 (Table 1). The hydrolysis of β-glycosidic linkages of agarose can produce NAOs of diverse sizes, including neoagarobiose (NA2), neoagarotetraose (NA4), neoagarohexaose (NA6),

Table 1 Agar-degrading enzymes with characteristic features

Enzyme type	Characteristic feature type	Bacterial species	Protein	Molecular mass (kDa)	Glycoside hydrolase family	Optimal temperature	Optimal pH	Degradation products	Activation by metal ions	Characteristic features	Reference
β-Agarase	Thermostability	*Halococcus* sp. 197A	Aga-HC	55	Not available	70	6.0	NA4, NA6	Not available	Retaining more than 50% of maximum activity after pre-incubation at 95 °C for 60 min	(Minegishi et al. 2013)
		Bacillus sp. BI-3	BI-3	58	Not available	70	6.4	NA2	Ca^{2+}, Mg^{2+}, Sr^{2+}, K^{+}, Na^{+}	Retaining more than 50% of maximum activity after pre-incubation at 80 °C for 15 min	(Li et al. 2014)
		Microbulbifer pacificus LD25	AgaL4	84.6	GH50	60	7.0	NA2	Ca^{2+}	Retaining 93% of maximum activity after pre-incubation at 70 °C for 180 min	(Chen et al. 2019c)
		Pseudoaltero-monas fuliginea YTW1-15-1	Aga0917	32.5	GH16	60	6.0	NA4	Co^{2+}, Mg^{2+}, K^{+}, Na^{+}		(Wang et al. 2019)
		Persicobacter sp. CCB-QB2	PdAgaC	37	GH16	60	7.5	NA2	None	Good thermostability at 55 °C	(Hafizah et al. 2019)
		Catenovulum agarivorans YM01	YM01-3	46.9	GH16	60	6.0	NA4, NA6	Na^{+}, K^{+}		(Cui et al. 2014)
		Gilvimarinus agarilyticus JEA5	Gaa16A	49	GH16	55	7.0	NA4	Ca^{2+}, Mn^{2+}, Fe^{2+}, Na^{+}	Retaining 85% of maximum activity at 65 °C	(Lee et al. 2018a)
		Flammeovirga sp.OC4	Aga4436	54	GH16	55	6.5	NA4, NA6	Co^{2+}, Mn^{2+}	Retaining 80% of maximum activity at 60 °C	(Chen et al. 2016)
		Microbulbifer sp. JAMB-A94	AgaA	48	GH16	55	7.0	NA4	None	Retaining more than 80% of maximum activity after pre-incubation at 60 °C for 15 min	(Ohta et al. 2004a)
		Pseudoaltero-monas sp. AG4	AgaP	72.5	GH16	55	5.5	NA4, NA6	Not available	Retaining more than 50% of maximum activity after pre-incubation at 55 °C for 60 min	(Oh et al. 2010)
			Agy1	69	GH16	55	7.6	NA4, NA6			

Table 1 (continued)

Enzyme type	Characteristic feature type	Bacterial species	Protein	Molecular mass (kDa)	Glycoside hydrolase family	Optimal temperature	Optimal pH	Degradation products	Activation by metal ions	Characteristic features	Reference
		Saccharophagus sp. AG21							Fe^{2+}, Na^{+}, K^{+}		(Lee et al. 2013)
		Paenibacillus agarexedens BCRC17346	AgaB-4	97	GH50	55	6.0	NA4	Co^{2+}, Mn^{2+}		(Chen et al. 2018)
		Microbulbifer sp. JAMB-A7	RagaA7	49	GH16	50	7.0	NA4	None	Having a half-life of 502 min at 50 °C	(Ohta et al. 2004b)
		Flammeovirga sp. HQM9	AgaDL6	52.8	GH16	50	3.0	NA4, NA6	Mg^{2+}, Ba^{2+}, Ca^{2+}, Co^{2+}, Na^{+}, Zn^{2+}	Retaining more than 98% of maximum activity after pre-incubation at 50 °C for 2 h	(Liu et al. 2019b)
		Metagenomic library	AgaM1	80	GH16	50	7.0	NA4, NA6	None	Retaining more than 70% of maximum activity after pre-incubation at 40 °C for 60 h	(Di et al. 2018)
		Paenibacillus sp. SSG-1	SSG-1a	77	Not available	50	6.0	NA6, NA8	Mn^{2+}		(Song et al. 2014)
		Cohnella sp. LGH	AgaW	97	GH50	50	7.0	Major: NA4 Minor: NA2	Ca^{2+}, Mg^{2+}, Na^{+}, K^{+}		(Li et al. 2015a)
		Metagenomic library	AgaML	71.6	GH16	50	7.0	NA4, NA6	Ca^{2+}, Mn^{2+}, Ba^{2+}, Co^{2+}, Na^{+}, K^{+}		(Mai et al. 2016)
		Catenovulum agarivorans YM01	YM01-1	37.3	GH16	50	7.0	NA2	Ca^{2+}, Mg^{2+}, K^{+}		(An et al. 2018)
		Microbulbifer sp. BN3	N3-1	34.3	GH16	50	6.0	NA2, NA4	Na^{+}, K^{+}		(Li et al. 2018)
		Metagenomic library	Aga2	37	GH16	50	7.0	n.d.	None		(Qu et al. 2018)
	Cold tolerance	*Gayadomonas joobiniege* G7	AgaJ9	134	GH39	25	5.0	NA2, NA4	None	Retaining 80% of maximum activity at 5 °C	(Jung et al. 2017b)
		Aquimarina agarilytica ZC1	Aga672	98	GH16	25	7.0	Major: NA4, NA6 Minor: NA8	None		(Lin et al. 2017)
			Aga21	80	Not available	30	8.0	NA2	Ca^{2+}, Mg^{2+}, Mn^{2+}, Ba^{2+},	Retaining 85% of maximum activity at 10 °C	(Li et al. 2015b)

Table 1 (continued)

Enzyme type	Characteristic feature type	Bacterial species	Protein	Molecular mass (kDa)	Glycoside hydrolase family	Optimal temperature	Optimal pH	Degradation products	Activation by metal ions	Characteristic features	Reference
		Pseudoalteromonas sp. NJ21							Zn^{2+}, Cu^{2+}, Co^{2+}, Fe^{2+}, Sr^{2+}, Na^{+}, K^{+}		
		Gayadomonas joobiniege G7	AgaJ5	89	GH86	30	4.5	Major: NA6 Minor: NA2, NA4	Na^{+}, K^{+}	Retaining 40% of maximum activity at 10 °C	(Lee et al. 2018b)
		Gayadomonas joobiniege G7	AgaJ10	89	GH42	30	5.0	NA2	Mn^{2+}	Retaining more than 40% of maximum activity at 5 °C	(Choi et al. 2019)
		Pseudoalteromonas sp. NJ21	Aga3463	40	GH86	50	7.0	NA2	Ca^{2+}, Cd^{2+}, Mn^{2+}, Fe^{2+}	Retaining more than 60% of maximum activity at 10 °C	(Li et al. 2019)
	Alkaline	*Stenotrophomonas* sp. NTa	β-agarase	89	Not available	40	10.0	Major: NA2, NA4, NA6 Minor: AHG	Mg^{2+}, Ba^{2+}, Ca^{2+}, Na^{+}, K^{+}	Striking stability across a wide pH range of 5.0–11.0	(Zhu et al. 2016)
		Flammeovirga sp. OC4	Aga3027	93	GH16	40	9.0	NA4, NA6	Mn^{2+}, Sr^{2+}	Retaining more than 60% of maximum activity at pH 10.0	(Chen et al. 2019a)
		Flammeovirga sp. SJP92	AgaB	106	GH16	45	8.0	NA4, NA6	None	Retaining 85% of maximum activity at pH 8.5–9.5	(Dong et al. 2016)
		Agarivorans albus YKW-34	AgaA34	50	GH50	40	8.0	NA2, NA4	None	Retaining more than 65% of maximum activity at pH 10.0–11.0	(Fu et al. 2008)
		Agarivorans sp. LQ48	AgaA	51	GH16	40	7.0	NA4, NA6	Mn^{2+}	Retaining more than 95% of maximum activity after pre-incubation at pH 3.0–11.0 for 1 h	(Long et al. 2010)
		Aquimarina agarilytica ZC1	Aga672	98	GH16	25	7.0	Major: NA4, NA6 Minor: NA8	None	Retaining more than 80% of maximum activity after pre-incubation at pH 7.0–11.0 for 1 h	(Lin et al. 2017)
			Aga4436	54	GH16	55	6.5	NA4, NA6	Co^{2+}, Mn^{2+}		

Table 1 (continued)

Enzyme type	Characteristic feature type	Bacterial species	Protein	Molecular mass (kDa)	Glycoside hydrolase family	Optimal temperature	Optimal pH	Degradation products	Activation by metal ions	Characteristic features	Reference
		Flammeovirga sp.OC4								Striking stability across a wide pH range of 3.0–11.0	(Chen et al. 2016)
		Paenibacillus sp. SSG-1	Aga1	165	GH86	40	5.0	NA4, NA6	Not available	Strong stability across a wide pH range of 2.0–11.0	(Song et al. 2016)
	Eosinophilic	*Flammeovirga* sp. HQM9	AgaDL6	52.8	GH16	50	3.0	NA4, NA6	Mg^{2+}, Ba^{2+}, Ca^{2+}, Co^{2+}, Na^{+}, Zn^{2+}	Retaining 100% of maximum activity after pre-incubation at pH 2.0–5.0 for 24 h	(Liu et al. 2019b)
		Gayadomonas joobiniege G7	AgaJ11	35	GH16	40	4.5	NA2, NA4, NA6	None	Active only at a narrow pH range from 4.5 to 5.5	(Jung et al. 2017a)
		Gayadomonas joobiniege G7	AgaJ5	89	GH86	30	4.5	Major: NA6 Minor: NA2, NA4	Na^{+}, K^{+}	Having strong activity only at a narrow pH range from 4.5 to 5.5	(Lee et al. 2018b)
		Gayadomonas joobiniege G7	AgaJ9	134	GH39	25	5.0	NA2, NA4	None	Retaining more than 70% of maximum activity at pH 4.0	(Jung et al. 2017b)
		Gayadomonas joobiniege G7	AgaJ10	89	GH42	30	5.0	NA2	Mn^{2+}	Retaining more than 70% of maximum activity at pH 4.0	(Choi et al. 2019)
		Flammeovirga sp.OC4	Aga4436	54	GH16	55	6.5	NA4, NA6	Co^{2+}, Mn^{2+}	Striking stability across a wide pH range of 3.0–11.0	(Chen et al. 2016)
		Pseudoalteromonas sp. Q30F	Aga862	50	GH16	45	6.5	NA4, NA6	Co^{2+}, Cu^{2+}, Zn^{2+}	Retaining more than 80% of maximum activity at pH 3.0–10.0	(Cui et al. 2018)
	Detergent-resistance	*Microbulbifer* sp. JAMB-A7	RagaA7	49	GH16	50	7.0	NA4	None	Strong resistance against 2% SDS	(Ohta et al. 2004b)
		Agarivorans albus YKW-34	AgaB34	49	GH16	30	7.0	NA4	None	Good resistance against SDS and urea	(Fu et al. 2009)
		Stenotrophomonas sp. NTa	β-agarase	89	Not available	40	10.0	Major: NA2, NA4, NA6 Minor: AHG	Mg^{2+}, Ba^{2+}, Ca^{2+}, Na^{+}, K^{+}	Good resistance against detergents and urea	(Zhu et al. 2016)
		Flammeovirga sp. SJP92	AgaB	106	GH16	45	8.0	NA4, NA6	None		(Dong et al.

Table 1 (continued)

Enzyme type	Characteristic feature type	Bacterial species	Protein	Molecular mass (kDa)	Glycoside hydrolase family	Optimal temperature	Optimal pH	Degradation products	Activation by metal ions	Characteristic features	Reference
										Good resistance against SDS and urea	2016)
		Microbulbifer sp. Q7	β-agarase	65	GH16	40	6.0	NA4, NA6	None	Good resistance against SDS and urea	(Su et al. 2017)
		Persicobacter sp. CCB-QB2	PdAgaC	37	GH16	60	7.5	NA2	None	Good resistance against several detergents	(Hafizah et al. 2019)
	High salt resistance	*Cellulophaga algicola* DSM14237	CaAga1	39	GH16	40	7.0	NA4, NA6	Mn^{2+}, Fe^{2+}, Ca^{2+}, Co^{2+}, Mg^{2+}	Retaining more than70% of maximum activity in the presence of 2 M NaCl	(Han et al. 2019)
	Specific degradation products	*Catenovulum* sp. X3	AgaXa	52	GH118	52	7.4	NA6, NA8, NA10, NA12	Mg^{2+}, Ca^{2+}, Na^{+}, K^{+}	The enzyme cannot degrade NA8 and smaller neoagarooligosaccharides.	(Xie et al. 2013)
		Vibrio sp. PO-303	AgaC	47	GH118	35	6.0	NA4, NA6, NA8, NA10, NA12	Not available	The enzyme cannot degrade NA8 and smaller neoagarooligosaccharides.	(Dong et al. 2006)
		Pseudoalteromonas sp. CY24	AgaB	51	GH118	40	6.0	NA4, NA6, NA8, NA10	Mg^{2+}, Ba^{2+}	The enzyme cannot degrade NA8 and smaller neoagarooligosaccharides.	(Ma et al. 2007)
		Agarivorans sp. JA-1	AgaJA2	52	GH118	35	7.0	NA8	Not available		(Lee et al. 2012)
	Others	*Gayadomonas joobiniege* G7	AgaJ9	134	GH39	25	5.0	NA2, NA4	None	Existing as two forms, dimer and monomer	(Jung et al. 2017b)
α-Agarase	Cold tolerance	*Catenovulum sediminis* WS1-A	AgaWS5	140	GH96	40	8.0	A4	Ca^{2+}	Retaining more than 40% of maximum activity at 10 °C	(Lee et al. 2019a)
	Alkaline	*Catenovulum agarivorans*	CaLJ96	200	GH96	37	7.0	A4	Ca^{2+}	Retaining more than 80% of maximum activity at pH 9.0–10.0	(Liu et al. 2019a)
	Cold tolerance		Ahg786	45	GH117	15	7.0		Mn^{2+}		

Table 1 (continued)

Enzyme type	Characteristic feature type	Bacterial species	Protein	Molecular mass (kDa)	Glycoside hydrolase family	Optimal temperature	Optimal pH	Degradation products	Activation by metal ions	Characteristic features	Reference
Neoagarooligosaccharide hydrolase		*Gayadomonas joobiniege* G7						AHG, NAOs		Retaining more than 70% of maximum activity at 4 °C	(Asghar et al. 2018)
		Cellulophaga sp. W5C	AhgI	45	GH117	20	7.0	AHG, NAOs	Ca^{2+}		(Ramos et al. 2017)
		*Cellvibrio*sp.-WU-0601	α-NAO hydrolase	42	GH117	25	6.0	AHG, NAOs	Mn^{2+}, Mg^{2+}	Retaining more than 80% of maximum activity at 20 °C	(Watanabe et al. 2016)
		Cytophagaflevensis	α-NABH	Not available	Not available	25	6.75	AHG, G	Not available		(Van Der Meulen and Harder 1976)

neoagarooctaose (NA8), neoagarodecaose (NA10), and neoagarododecaose (NA12); however, the hydrolysis products by β-agarases varies according to the type of β-agarase. Among the many characterized β-agarases, we have summarized the ones with unique characteristic features suitable for industrial applications in Table 1.

Agar powder melts to the sol state upon heating to 90–95 °C, and the transition from the sol state to the gel state occurs upon cooling to 30–40 °C (Normand et al. 2000). Because agarases cannot efficiently degrade agar in the gel state (Kim et al. 2017b), agarases with maximum activity at high temperature or with high thermostability are suitable for industrial applications. Aga-HC sourced from a halophilic archaeon *Halococcus* sp. 197A showed maximum activity at 70 °C and retained more than 50% of this activity after pre-incubation at 95 °C for 60 min (Minegishi et al. 2013). However, the nucleotide and amino acid sequences of Aga-HC were not determined, and thus, its GH family was also not determined. Aga-HC is the only agarase purified from Archaea. Agarase BI-3, obtained from a thermophilic bacterium *Bacillus* sp. BI-3 isolated from hot spring, also exhibited maximum activity at 70 °C, but its thermostability was relatively worse than that of Aga-HC. BI-3 retained more than 50% of its maximum enzymatic activity after pre-incubation at 80 °C for 15 min, but its activity sharply decreased to less than 20% after pre-incubation for 30 min (Li et al. 2014). Similar to Aga-HC, the nucleotide and amino acid sequences of BI-3 have not been determined. Four β-agarases with maximum activity at 60 °C have been biochemically characterized and are listed in Table 1. Particularly, AgaL4 belonging to GH50 family from *Microbulbifer pacificus* LD25 isolated from a saltwater hot spring showed a high thermostability. It retained 93% of its maximum activity after pre-incubation at 70 °C for 180 min (Chen et al. 2019c). Many β-agarases with maximum activity at 50–55 °C have been biochemically characterized and they belong to the GH16 or GH50 family (Table 1). Because the transition from the sol state to the gel state of agar occurs upon cooling to less than 40 °C, β-agarases with maximum activity at more than 50 °C are suitable for industrial applications. However, the transition of agar powder to the sol state requires heating to more than 90 °C; therefore, identification of agarases with better thermostability would be of more useful in industrial applications.

Several β-agarases exhibiting cold tolerance have also been biochemically characterized (Table 1). AgaJ9, isolated from an agar-degrading marine bacterium *Gayadomonas joobiniege* G7, exhibited strong cold tolerance (Jung et al. 2017b). It showed maximum activity at 25 °C and retained 80% of its activity at 5 °C. The efficiency of AgaJ9 in DNA extraction from agarose gel at room temperature was examined. AgaJ9 completely recovered the DNA from 0.5% agarose gel at room temperature after melting of the gel. It also partially recovered the DNA from the gel state of agarose at

room temperature (Jung et al. 2017b), indicating that AgaJ9 can partially degrade agarose in the gel state. Besides thermostable agarases, agarases that can efficiently degrade agarose in the gel state also have a strong potential for industrial applications. AgaJ9 has some other characteristic features as well. It is the only agarase belonging to the GH39 family and exists in two forms, dimer and monomer, both having similar enzymatic properties (Jung et al. 2017b). Additionally, AgaJ9 is an eosinophilic β-agarase with maximum activity at pH 5.0 and retains more than 70% of maximum activity at pH 4.0. Similar to AgaJ9, many agarases from *G. joobiniege* G7 showed the cold tolerance (Jung et al. 2017b; Asghar et al. 2018; Lee et al. 2018b; Choi et al. 2019) and eosinophilic properties (Jung et al. 2017a; Jung et al. 2017b; Lee et al. 2018b; Choi et al. 2019). Additionally, AgaJ9 and AgaJ10 from *G. joobiniege* G7 are the first β-agarases belonging to the GH39 and GH42 families, respectively (Jung et al. 2017b; Choi et al. 2019). Therefore, *G. joobiniege* G7 may be an interesting agar-degrading bacterium with various unique agarases.

Many alkaline or eosinophilic β-agarases have been biochemically identified (Table 1). β-Agarase, obtained from a marine bacterium *Stenotrophomonas* sp. NTa exhibited maximum activity at pH 10.0 and retained 32% of it at pH 11.0 (Zhu et al. 2016). However, the nucleotide and amino acid sequences of this enzyme were not determined. A recent study showed that Aga3027 from *Flammeovirga* sp. OC4 isolated from the deep sea displayed high pH stability (Chen et al. 2019a). Aga3027 belonging to the GH16 family exhibited maximum activity at pH 9.0 and retained more than 60% of it at pH 10.0. Although other β-agarases displaying high stability in pH ranging from 9.0 to 11.0 have been identified, all of them showed maximum activity at a pH lower than pH 8.0. Several interesting eosinophilic β-agarases have been also identified. A GH16 β-agarase, AgaDL6, isolated from a marine agar-degrading bacterium *Flammeovirga* sp. HQM9, showed maximum activity at pH 3.0 and retained approximately 96% of maximum activity even at pH 2.0 (Liu et al. 2019b). AgaJ11 and AgaJ5 from *G. joobiniege* G7 are also eosinophilic β-agarases that exhibit maximum activity at pH 4.5 and strong activity only at a narrow pH range from 4.5 to 5.5 (Jung et al. 2017a; Lee et al. 2018b). Other β-agarases having maximum activity in pH ranging from 5.0 to 6.5 are summarized in Table 1. For industrial processes such as biofuel production, which require total hydrolysis of agar, use of eosinophilic β-agarases together with acid treatment under mild conditions would be efficient.

Detergent-resistance and high salt-resistance of agarases may also be useful in industrial application (Table 1). A GH16 β-agarase, RagaA7, obtained from an agar-degrading bacterium *Microbulbifer* sp. JAMB-A7, exhibited strong resistance against sodium dodecyl sulfate (SDS) (Ohta et al. 2004a). It showed the 1.4-fold increased activity after pre-incubation for 1 h in 0.1% SDS compared with that of the control (without SDS) and maintained more than 90% of its maximum activity after pre-incubation for 1 h in 2% SDS. RagaA7 also displayed strong resistance to EDTA up to a concentration of 100 mM (Ohta et al. 2004a). A recent study showed that a GH16 β-agarase, PdAgaC, obtained from a marine agarolytic bacterium *Persicobacter* sp. CCB-QB2, also shows 1.24-fold higher activity in 0.1% SDS and Triton X-100 treatment than without SDS (Hafizah et al. 2019). A GH16 β-agarase, CaAga1, sourced from a marine bacterium *Cellulophaga algicola* DSM 14237, showed high salt tolerance that retained more than 70% of maximum activity in the presence of 2 M NaCl (Han et al. 2019).

Most β-agarases degrade agarose into NA2, NA4, NA6, or mixtures of these NAOs (Table 1). Several β-agarases can degrade agarose into NAOs with the sizes larger than NA6 (Table 1). Because most biological activities of NAOs depend on their size (Kobayashi et al. 1997; Hong et al. 2017a; Kim et al. 2017a), the search of β-agarases that can produce NAOs with the sizes larger than NA6 is important. AgaXa from *Catenovulum* sp. X3 degraded agarose into NA6, NA8, NA10, and NA12 (Xie et al. 2013). Similarly, AgaC obtained from a marine bacterium *Vibrio* sp. PO-303 also degraded agarose into NA4, NA6, NA8, NA10, and NA12 (Dong et al. 2006), and AgaB from a marine bacterium *Pseudoalteromonas* sp. CY24, degraded agarose into NA4, NA6, NA8, and NA10 (Ma et al. 2007). None of these three β-agarases can degrade NA8 and smaller NAOs. Notably, these enzymes belong to the GH118 family. Based on the analyses of enzymatic kinetics and degradation patterns of different oligosaccharides by AgaB, the authors suggested that AgaB has a large substrate-binding cleft that accommodates 12 sugar units (Ma et al. 2007). Although crystallization and preliminary X-ray analysis of AgaB were performed (Ren et al. 2010), a three-dimensional structure of AgaB has not been resolved yet.

α-Agarases

Only 5 α-agarases hydrolyzing α-glycosidic linkages have been biochemically characterized (Potin et al. 1993; Ohta et al. 2005; Zhang et al. 2018; Lee et al. 2019a; Liu et al. 2019a). All characterized α-agarases belong to the GH96 family and degrade agarose into agarotetraose (A4). Their activity is enhanced in the presence of Ca^{2+}. As listed in Table 1, AgaWS5 from *Catenovulum sediminis* WS1-A showed cold tolerance (Lee et al. 2019a). It maintained more than 40% of its maximum activity at 10 °C. CaLJ96 from *C. agarivorans* is an alkaline α-agarase that retains more than 80% of its maximum activity at pH 9.0–10.0 (Liu et al. 2019a). Because only a few α-agarases were biochemically characterized, further studies are required to identify novel α-agarases with different

properties or producing different AOs, such as agarobiose (A2) or agarohexaose (A6).

α-Neoagarooligosaccharide hydrolases

Various α-neoagarooligosaccharide hydrolases (α-NAOSHs) have been biochemically characterized so far. According to scientific concepts, α-NABHs cleaves AHG of only NA2, whereas α-NAOSHs cleave AHG of various NAOs, including NA2, NA4, and NA6 (Table 1). Till date, two α-NABHs have been reported. However, their genetic information has not been uncovered (Day and Yaphe 1975; Van Der Meulen and Harder 1976). All characterized α-NAOSHs belong to the GH117 family (Asghar et al. 2018). Several cold-tolerant α-NAOSHs have been biochemically characterized. Ahg786 from *G. joobiniege* G7 exhibited maximum activity at 15 °C and retained more than 70% of its maximum activity at 4 °C (Asghar et al. 2018). Several α-NAOSHs with an optimal temperature ranging from 20 to 25 °C were also identified (Table 1).

The biological activities of agar and agar-derived oligosaccharides or mono-sugars

Applications of agar and agarose

Agar is a type of hydrophilic colloidal polysaccharide with unique properties of gel-sol transition on heating and cooling (Brinker and Scherer 1990; Renn 1997). Owing to its stabilizing and gelling ability, it has been widely used as a food additive, solidifying reagent in microbial culture media, and supporting materials for electrophoresis and various chromatographic procedures.

Recently, agar has also been spotlighted by many scientists because it is a renewable natural resource that can substitute fossil-based chemical feed stocks including petroleum. Moreover, it has several industrial applications. It can be used in enzyme or bacteria immobilization techniques for improving biological processes. For example, β-galactosidase of *Aspergillus oryzae* was immobilized in an agar matrix and successfully used for synthesizing lactulose (Guerrero et al. 2017). The photosynthetic bacterial cells of *Rhodobacter capsulatus* DSM 1710 (wild type) and YO3 (hup-mutant) strains were immobilized in an agar matrix and applied in sequential batch process for biological hydrogen production (Elkahlout et al. 2017), which resulted in a markedly prolonged hydrogen production time from 17 days up to 60 days. Because immobilization of enzyme and viable cells in a non-toxic agar can enhance the stability of the system and thus allow long-term operation, developing agar-based immobilization bioprocess can offer a superior advantage over other supporting materials.

Secondly, agar can be used for preparing biodegradable polymers such as wet-fiber (Liu et al. 2018), polyvinyl alcohol (PVA)-agar films (Madera-Santana et al. 2011), poly(2-hydroxyethylmethacrylate) grafted agar (Rani et al. 2013), or bioplastics (Jumaidin et al. 2016). Addition of agar to 30% (wt/wt) could greatly improve the shortcomings of starch-based thermoplastic that is manufactured by hot pressurized process at 140 °C for 10 min by enhancing tensile properties, thermal stability, and moisture uptake (Jumaidin et al. 2016). Therefore, agar can be used to produce eco-friendly bioplastics as a substitute for the traditional recalcitrant plastics for short-life products such as packaging. The use of agar has also been reported in an eco-friendly, biocleaning process (Ranalli et al. 2019). An agar-gauze biogel system activated with viable *Pseudomonas stutzeri* cells was successfully applied for onsite cleaning and restoration of altered historical wall paintings.

Thirdly, agar can be used in medical treatment such as drug delivery and microencapsulation. The agar hydrogel composite as an antibiotic delivery system could significantly improve the treatment efficacy toward microbial infections of skin and soft tissue (Pandit et al. 2018). Use of agarose-based microencapsulation system could increase the antifungal activity of gallic acid, but decrease cytotoxicity by *Aspergillus niger* in human skin keratinocyte (Lam et al. 2015). Recently, a highly macroporous bone scaffold was prepared using a polysaccharide matrix composed of chitosan and agarose and applied in bone generation (Kazimierczak et al. 2019). The novel agarose-containing biomaterial showed no toxicity to osteoblasts and promoted cell attachment and proliferation on its surface, which is an impressive result for application of agar in regenerative medicine. Agar has several properties that make it suitable for medical use. It has theranostic (therapeutic/diagnostic) properties, physical and chemical stability, adjustable softness, ease of application and removal for wound area, and no known toxicity. In this context, it has a high potential for application in various medical fields such as wound treatment and regenerative therapies.

Neoagarooligosaccharides and metabolic syndrome

Globally, the prevalence of obesity is increasing due to environmental factors such as dietary habits and changes in modern lifestyle. It is an important cause of insulin resistance, which impairs insulin sensitivity and increases the incidence of type 2 diabetes mellitus (T2DM) (Kahn et al. 2006; Ruan and Dong 2016). Obesity increases the secretion of most adipocytokines in adipocytes, which are known to regulate total energy metabolism including lipid and glucose metabolism. This results in hyperlipidemia and fatty liver (Trayhurn 2005; Ghadge et al. 2018). Recently, we reported anti-obesity

and anti-diabetic effects of neoagarooligosaccharides (NAOs), which are composed of a mixture of NA4 and NA6, prepared by enzymatic hydrolysis of commercial agar with DagA β-agarase (Hong et al. 2017a). Intake of NAOs (0.5% w/w) for 67 days showed a notable reduction in body weight gain by 36% without causing any abnormal clinical signs in the high-fat diet (HFD)–fed obese mice. Moreover, we could demonstrate a similar effect in a rat model (Fig. 2). Intake of NAOs (0.5% w/w) for long period of 151 days resulted in much significant reduction of body weight gain by 46.9% compared to that of the HFD group (Fig. 2). Other indicators of metabolic syndrome resulting from obesity such as adipose tissue development, hyperlipidemia, steatosis, insulin resistance, and glucose intolerance, were markedly improved, and adiponectin level in blood were recovered by intake of NAOs in both the animal models. Since insulin resistance, hyperlipidemia, and fatty liver are all secondary causes of obesity, improvement in obesity by intake of NAOs was expected to ameliorate all the above symptoms (Kahn et al. 2006).

Adiponectin is abundant and circulates in human plasma in concentrations ranging from 2 to 20 mg/mL, with a broad-range of biological activities such as improving insulin sensitivity in major insulin target tissues, modulating inflammatory responses, and controlling homeostasis in energy and glucose metabolism (Turer and Scherer 2012; Ruan and Dong 2016). It facilitates fat storage in smaller adipocytes and enhances insulin-stimulated glucose uptake in adipocytes. Moreover, it suppresses the biosynthesis of glucose, triglyceride, ceramide, and lipogenesis in liver, while enhancing fatty acid oxidation and insulin-stimulated glucose utilization in skeletal muscle by stimulating the AMPK pathway (Ghadge et al. 2018). In pancreas, it promotes β-cell function and survival (Turer and Scherer 2012). Therefore, when the concentration of adiponectin is restored, most symptoms of insulin resistance induced by obesity are expected to be improved, and the favorable results of our study may be attributed to the adiponectin concentration recovered by ingestion of NAOs (Hong et al. 2017a), which controls adipogenesis via adiponectin signaling pathway by suppressing the transcription of master switches, resulting in less lipid accumulation and weight gain.

Recently, Fudi Lin and colleagues reported that NAO intake (400 mg/kg body weight) for 11 weeks could prevent weight loss in T2DM mice model established by the combination of a HFD and alloxan injection to similar level treated with anti-diabetic drug metformin (50 mg/kg) (Lin et al. 2019), indicating it can be also used for treating T2DM. Moreover, NAO improved metabolic disorders such as blood glucose level, glucose tolerance, hepatic macrovesicular steatosis, pancreatic β-cells defects, and enhanced oxidative stress enzymes including glutathione peroxidase and

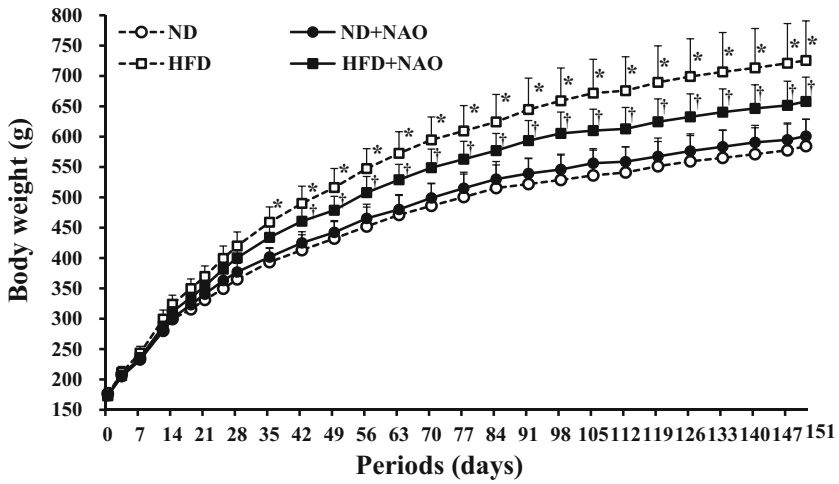

Fig. 2 Effects of NAO on body weight changes in Sprague Dawley (SD) rat fed with high-fat diet for 151 days. The experiment was performed as previously described (Hong et al. 2017a) in SD rats (8 rats/group), at Gyeonggi Bio Center (Republic of Korea) with the approval of Institutional Animal Care and Use Committee (permission number of 2013-05-121). The experiment start date was designated as day 0, and the body weight was measured immediately before beginning the experiment. The NAO was prepared directly from red algae. The dried algal biomass of *Gelidium elegans* Kützing (Jejuseagreen Co. Ltd., Jeju, Korea) was immersed in distilled water at a concentration of 2 to 3% (w/v), and the agar was extracted by heating it (90 to 100 °C, 4 h). DagA β-agarase was prepared by culturing *Streptomyces coelicolor* A3(2) M22-2C43 as previously described (Hong et al. 2019). The enzymatic reaction was carried out at 43 °C with an agitation of 30 rpm by adding 0.1 to 0.2 U/mL of crude DagA enzyme to the 2.5% agar solution. After 16 h, the enzyme was inactivated by heat treatment at 95 °C with an agitation of 30 rpm for 2 h. The product was then concentrated 20 times in vacuo (65 °C, 1.3 kpa, 3 h) and finally lyophilized using freeze dryer to obtain a powder of NAO test material. The purity of NAO obtained was approximately 55% and the main components were found to be neoagarotetraose (33.6%) and neoagarohexaose (21.6%). ND normal diets, ND + NAO normal diets + 0.5% (w/w) neoagarooligosacchrides, HFD high-fat diet, HFD + NAO high-fat diet + 0.5% (w/w) neoagarooligosacchrides. Each data was presented as mean ± SD ($n = 8$). * represents a significant difference at $p < 0.05$ level versus the ND group. † represents a significant difference at $p < 0.05$ level versus HFD group

superoxide dismutase. NAO activated mitogen-activated protein kinase (MAPK) pathway by suppressing the phosphorylation of ERK1/2 and JNK that caused hepatic injury, and enhanced the expressions of a basic leucine zipper protein Nrf2 (nuclear factor erythroid-2-related factor 2) and antioxidant proteins (oxygenase 1 and NAD(P)H quinone dehydrogenase 1) that are regulated by Nrf2 involved in oxidative stress in T2DM mice model.

The effect of NAO intake on maintaining cholesterol homeostasis was reported in mice model fed on a high-cholesterol diet (HCD) (Yang et al. 2017). NAOs could recover LDL receptor (LDLR) by enhancing its expression and resulted in increased LDL uptake in hepatocytes of HCD-mice. It particularly stimulated the phosphorylation of Akt (PKB) and its substrate, glycogen synthase kinase 3β (GSK3β), which in turn activates SREBP-2, a crucial regulator for LDLR gene expression and cholesterol metabolism, by phosphorylation.

Our in vitro experiment using mouse 3T3-L1 cells revealed that NAOs can effectively suppress adipogenesis and lipid accumulation (unpublished data). Moreover, real-time PCR analysis demonstrated that NAOs inhibited the transcription of the key adipogenic transcription regulators such as sterol regulatory element binding protein 1C (SREBP-1c), PPAR-γ, and CCAAT/enhancer binding protein α (C/EBP-α).

PI3K/AKT pathway is one of the components in insulin signaling pathway and regulates most metabolic effects endowed by insulin. The main protein involved in adiponectin signaling pathway, APPL1, is also known to be involved in insulin signaling pathway by promoting IRS1/2 binding to the insulin receptor (Ruan and Dong 2016). Therefore, based on all the results available so far, the effect of NAOs on metabolic syndrome is illustrated in Fig. 3. In conclusion, NAOs seems to act comprehensively on the adiponectin, insulin, and MAPK signaling pathways in cells to improve metabolic abnormalities including cholesterol homeostasis and oxidative stress caused by obesity and diabetes.

Many anti-obesity drugs have been developed and approved by the US Food and Drug Administration for treatment of obesity. Majority of the drugs, such as phentermine and bupropion, decrease appetite by boosting central norepinephrine release. However, they have common adverse effects such as dizziness, dry mouth, insomnia, paresthesia, constipation, and increased heart rate and blood pressure, limiting their long-term use for more than 3 months (Burguera et al. 2017). Unlike these, orlistat inhibits pancreatic and gastric lipases and thus, decreases fat absorption. However, it has reported side effects such as flatulence with discharge and diarrhea after ingestion of high-fat diet as well as kidney damage have been reported (Acharya et al. 2006). Therefore, it is the need of the hour to develop a more effective anti-obesity agent with less toxicity, which has a pharmacological action different from that of the conventional drugs. In the safety evaluation, fortunately, no observed adverse effect level (NOAEL) was observed in rats administered with a dosage of 5000 mg/kg body weight/day of NAOs in the 90-day repeated oral dose toxicity tests (Hong et al. 2017b). Therefore, all the data clearly supported NAOs as a non-toxic material with excellent metabolic disease-improving effects on obesity and T2DM.

Neoagarooligosaccharides and immune reaction

According to recent reports (Park et al. 2014; Kang et al. 2017; Lee et al. 2017; Wang et al. 2017), NAO is a suitable candidate for pharmaceutical applications with excellent immunomodulatory activity. For example, organ damage and neutrophil infiltration in an LPS-induced endotoxemia and *Escherichia coli* K1-induced septic shock mouse model were markedly prevented by NAOs intake (Kang et al. 2017). Immunoblotting analysis of dendritic cells (DCs) treated with NAOs indicated that the enhancement of interleukin-10 secretion via the induction of A20 and COX-2 by NAOs treatment may be responsible for its preventive effect in septic shock.

NA4 showed significant anti-inflammatory effect in lipopolysaccharide (LPS)-stimulated mouse macrophage RAW264.7 (Wang et al. 2017). It lowered the cellular level of nitric oxide (NO) by suppressing the expression of inducible nitric oxide synthase (iNOS) as well as pro-inflammatory cytokines including IL-6 and TNF-α. The suppression of the activation of MAPK (p38MAPK and Ras/MEK/ERK) and NF-κB signaling pathways seems to be responsible for anti-inflammatory activity of NAOs in LPS-stimulated macrophages.

Antitumor activity of NAOs was also reported by using a mouse model that was subcutaneously inoculated with B16F1 melanoma cells (Lee et al. 2017). Intraperitoneal administration of NA6 (100 and 500 mg/kg of body weight) showed significant inhibition of tumor in the mouse model and this antitumor activity was associated with stimulation of DC mediated-natural killer cells through Toll-like receptor 4 (TLR4). Moreover, crucial factors for TLR4-mediated DC activation such as MAPK, AKT, and NF-κB were activated by NAOs in DCs in a TLR4-dependent manner (Park et al. 2014).

In fact, the expression of A20 and COX-2 is known to be stimulated through MAPK and NF-κB-dependent pathways (Kang et al. 2017). In these senses, the preventive effect of septic shock, and anti-inflammatory and antitumor activities of NAO seem to share similar signaling pathways.

Other biological activities of neoagarooligosaccharides

Agar-derived oligosaccharides have been reported to have various biological activities such as antioxidant activity, and skin-whitening and skin-moisturizing effects (Yun et al.

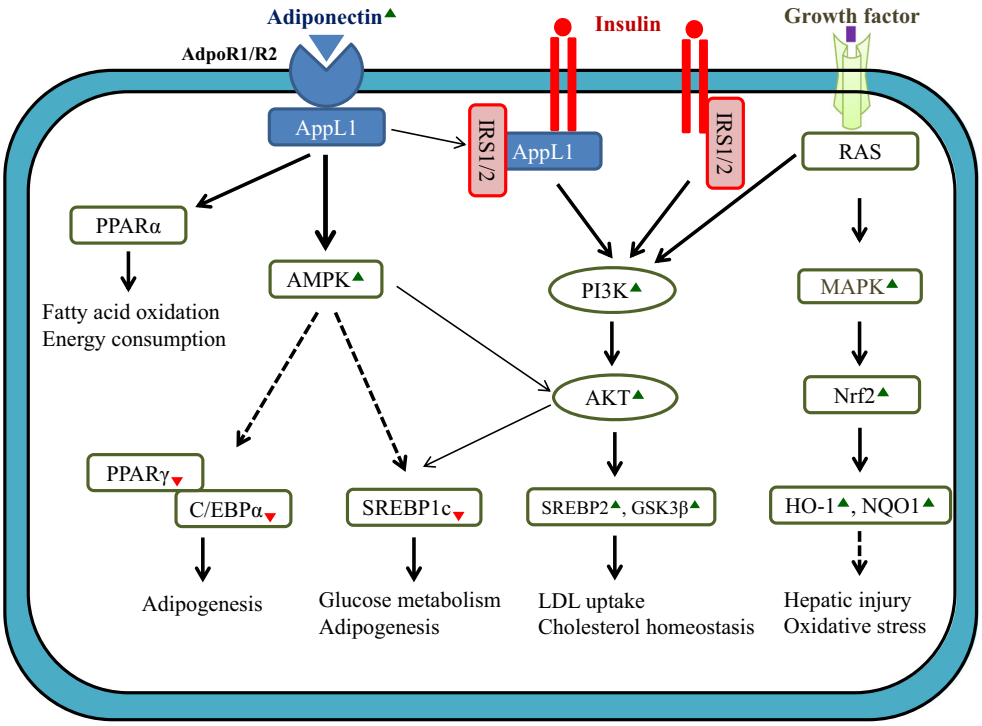

Fig. 3 Simplified schematic representation of the adiponectin, insulin, and mitogen-activated protein kinase (MAPK) signal transduction pathways for metabolic homeostasis. The signaling transduction cascades may begin with the activation of insulin and adiponectin receptors by their respective ligand binding. Binding of adiponectin to the AdipoR1 or AdipoR2 receptor activates APPL1 intracellular regulator, which triggers signal transduction route including AMPK that suppresses adipogenesis and glucose utilization. Signaling by insulin is mediated mainly by the PI3K/AKT pathway, leading to an increase in lipogenesis, LDL-uptake and cholesterol homeostasis, glucose uptake and utilization, and glycogen biosynthesis, but decrease in lipolysis and gluconeogenesis. APPL1, which is crucial for adiponectin signal transduction pathways also promotes binding IRS1/2 to the insulin receptor by forming the APPL1-IRS1/2 complex, which may enhance insulin signaling transduction. The MAPK signal transduction pathway is also involved in the physiological metabolic regulation of the cell. Moreover, the three signaling systems also seem to communicate through the AKT-AMPK, AKT-SREBP1c, and AKT-RAS pathway, and further research is needed to elucidate the underlying elaborate regulatory system between them. The bold line, dotted, and thin arrows indicate activation, suppression, and crosstalk (activation) relationship, respectively. In each signal transduction pathway, components found to be affected by NAO were selected and labeled with green triangles (activation) or inverted red triangles (suppression). AdipoR1 adiponectin receptor 1, AdipoR2 adiponectin receptor 2, AKT protein kinase B, APPL1 adaptor protein, phosphotyrosine interacting with PH domain and leucine zipper 1, AMPK 5′ adenosine monophosphate activated protein kinase, PPAR peroxisome proliferator activated receptor, SREBP sterol regulatory element binding protein, C/EBP-α CCAAT/enhancer binding protein α, GSK3β glycogen synthase kinase 3β, Nrf2 nuclear factor erythroid-2-related factor 2, HO-1 oxygenase 1, NQO1 NAD(P)H quinone dehydrogenase 1

2013). The skin-whitening activity of NA2 by inhibiting melanogenesis was reported in murine B16 melanoma cells (Kobayashi et al. 1997; Lee et al. 2008; Yun et al. 2013). At a concentration of 100 μg/mL, NA2 as well as AHG showed significantly lower melanin production compared to arbutin (Yun et al. 2013). At a concentration of 50 μg/mL, among the agar-derived saccharides tested, AHG showed the strongest skin-whitening activity in both murine B16 melanoma cells and human epidermal melanocytes, followed by NA4 and NA6, while NA2 and other AOs (agarotriose, agaropentaose, agaroheptaose) did not show any significant skin-whitening activity (Kim et al. 2017a). AHG did not show significant cytotoxicity up to 200 μg/mL in cell proliferation assays; therefore, it can be potentially used as a natural raw material in skin-whitening cosmetics. In conclusion, AHG appears to have a whitening effect only on oligosaccharides present at the non-reducing ends of NAOs (Yun et al. 2013).

NA4 showed the strongest hydroxyl radical scavenging activity among NA2, NA4, NA6, and NA8. NA2 has been reported to have a higher moisturizing power than glycerol and hyaluronic acid (Kobayashi et al. 1997) and have a higher inhibitory activity toward α-glucosidase (yeast maltase) than NA4 and NA6 (Hong et al. 2017c).

Recently, the anti-fatigue effects of NA4 in mice under intense exercise stress have been reported (Zhang et al. 2017). Intake of NA4 for 16-day resulted in a profound change in microbiome composition and increase of total short-chain fatty acids in the fecal matter. NA4 was effective in relieving intense exercise-induced liver injury by lowering liver weight, ALT and endotoxin (LPS) levels, and improving the liver morphological structure and bile acid level (Chen et al. 2019b). This is consistent with the results obtained in HFD-fed obese mice (Hong et al. 2017a). Therefore, NAO could be a potential prebiotic or hepatic function recovery agent.

Biological activities of agarooligosaccharides

The effects of AOs prepared by acid hydrolysis of agar on in vitro immunological reaction was reported in lipopolysaccharide (LPS)-stimulated RAW264.7 mouse macrophages and human monocytes (Enoki et al. 2010). According to the reports, the AOs (agarobiose, agarotetraose, and agarohexaose) significantly suppressed the cellular levels of nitric oxide, prostaglandin E_2, and pro-inflammatory cytokines including tumor necrosis factor-α, interleukin-1β, and interleukin-6 in both type of cells, which probably resulted from heme oxygenase-1 induction. Intake of AOs (0.3 g/kg/day) prevented tumor promotion on the two-stage mouse skin carcinogenesis model (Enoki et al. 2012) and suppression of prostaglandin E_2 production by AOs intake was proposed to be responsible for preventing tumor promotion.

Recently, a profound suppressive effect of AHG (36% suppression at 100 μg/mL and 62% suppression at 200 μg/mL) on nitrite production in LPS-stimulated RAW264.7 cells was reported, which indicates that AHG can be developed as an anti-inflammatory agent (Yun et al. 2013). 3,6-Anhydro-D-galactose, an enantiomer of AHG, also showed a nitrite-suppressing effect only at 200 μg/mL, but its effect was significantly lower than that of AHG. Contrastingly, NAOs (NA2 and NA6) did not show any nitrite-suppressing effect. Therefore, positioning AHG at the reducing ends of AOs was proposed to be the key for its anti-inflammatory activity (Enoki et al. 2012; Yun et al. 2013).

AHG and AOs including agarobiose (Yu et al., 2019) was recently suggested as an anti-cariogenic sugar to replace widely used xylitol, owing to its higher inhibitory activity against the growth and lactic acid production of *Streptococcus mutans*. However, NAOs including NA2 showed no inhibitory activity against the growth of *S. mutans*. Therefore, this inhibitory effect of AOs seems to be structure-specific to position AHG at their reducing ends.

Biofuel production from agar

Red macroalgae is considered as an alternative source of biomass for biofuel production, because of low contents of lignin or hemicellulose and high contents of carbohydrates (Yun et al. 2016). The major mono-sugars of red macroalgal biomass are galactose and AHG from agars and carrageenans and glucose from cellulose. Establishment of effective saccharification is necessary for production of biofuel from macroalgal biomass. Although acid hydrolysis cleaving the α-1,3-linkages of agarose is a rapid and cheap method, this method could produce inhibitors of the microbial fermentation, such as 5-hydroxymethylfulfural and levulinic acid, by the degradation of acid-labile AHG (Park et al. 2012). Therefore, enzymatic saccharification using agarases can be an alternative method.

The hydrolysis of agar using β-agarases and α-NABH produced galactose and AHG from agar (Ekborg et al. 2006; Hehemann et al. 2012; Yun et al. 2016). However, titers and yields of saccharification were low, due to the low solubility of agarose. If agarases that can efficiently degrade the agarose of the gel state are identified, they can be used for enzymatic saccharification. The efficiency of enzymatic saccharification was enhanced by chemical liquefaction of agarose through mild acid treatment, such as acetic acid (Kim et al. 2012; Lee et al. 2014; Lee et al. 2019b). Eosinophilic and thermostable agarases are more effective for these combined methods (Table 1). However, although chemical liquefaction and enzymatic saccharification of agarose efficiently produce monomeric sugars, a trace of agarotriose (G-AHG-G) was not degraded into monomeric sugars (Kim et al. 2012; Kim et al. 2013; Lee et al. 2014). Agarotriose could be completely hydrolyzed by the novel agarolytic β-galactosidase releasing G from AOs including agarotriose (Lee et al. 2014). Another report showed that the secretory overexpression of two β-agarases and one α-NABH from a plasmid in yeast induced saccharification of agarose and subsequently fermentation of monomeric sugars (Lee et al. 2019b). This industrial yeast strain produced a maximum of 1.97 g/L ethanol from 10 g/L agarose. In this system, traces of NA2 and NA6, but not agarotriose, remained after saccharification of agarose. These results imply that establishment of the most efficient method of saccharification using various effective agarases is still required.

There are two important obstacles for the efficient utilization of monomeric sugars (AHG, galactose, glucose) from red macroalgae. First, AHG is a non-favorable sugar for general microorganisms, despite being the most abundant sugar among monomeric sugars from red macroalgae. This problem was resolved by identification of two cytosolic enzymes, 3,6-anhydro-α-L-galactose dehydrogenase and 3,6-anhydrogalactonate cycloisomerase, which are necessary for the introduction of AHG into a general catabolic pathway (Yun et al. 2015). Introduction of these two enzymes into an industrial *Escherichia coli* KO11 strain showed a 24% increase in ethanol production from monomeric sugars of agarose, galactose and AHG (Yun et al. 2015). Secondly, glucose that may be produced by hydrolyzing cellulose of red algae is the most favorable sugar in most organisms and induces catabolite repression of galactose, which results in the inefficient consumption of galactose. Various attempts to increase the efficiency of galactose consumption were performed. In yeast, deletion of negative regulators of galactose metabolism (GAL6, GAL80, and MIG1) (Ostergaard et al. 2000), overproduction of phosphoglucomutase (PGM2) increasing the flux through the Leloir pathway (Bro et al. 2005), and overproduction of a small nuclear RNA SNR84 and a global transcriptional repressor TUP1 (Lee et al. 2011) improved the galactose utilization. Despite these various studies, a high

efficiency of galactose utilization in the presence of glucose has still not been achieved.

Recently, a study of production of biohydrogen from agar has been reported (Wu et al. 2017). Agar was saccharified with agarase (AgaXa) and NABH (NH852) into monomers, and then *Enterobacter* sp. CN1 was cultivated to produce hydrogen from the hydrolysate. Consequently, the application of enzymatic saccharification produced approximately 3.86 times more hydrogen than the control without the saccharification treatment. However, there is a persistent problem that the strain could not use AHG, but only galactose for hydrogen production. Therefore, engineering the industrial strain to efficiently utilize the non-favorable sugars, galactose and AHG, is necessary for biofuel production using red macroalgal biomass.

Conclusion

Agar has been traditionally used as a valuable material in industrial and laboratory applications, due to its gelling and stabilizing properties. Studies regarding the biological functions of agar-derived oligosaccharides first started in AOs produced by acid treatment, and subsequent studies were focused on NAOs or mono-sugars produced by the enzymatic hydrolysis of various agarases. NAOs particularly exhibited various unexpected biological activities, such as anti-obesity, anti-diabetic, immunomodulatory, antioxidant, skin-whitening, skin-moisturizing, and liver-protective and anti-fatigue activities. The molecular bases of these effects were partially determined, especially those underlying in the anti-obesity, anti-diabetic, and immunomodulatory effects. In general, since metabolic regulation and immune response regulation are closely linked to each other through a complex signaling system, it is not unusual that NAOs exert ameliorating effects on both metabolic syndrome and immune response. However, more detailed analyses on molecular basis of NAO effects are required for their pharmaceutical application. As the possibility of pharmaceutical application of NAOs increases, the necessity for identifying potent enzymes suitable for industrial application also increases. Although many diverse agarases have been biochemically characterized, none of agarases can efficiently degrade the agar in the gel or powder state, necessitating the melting of agar powder to the sol state upon heating to 90–95 °C, and then degrading it in the sol state. Therefore, identification of novel agarases that are not bound by these limitations is required. Novel agarases will be also of great assistance to enzymatic saccharification of agar for biofuel production. Therefore, agar-derived saccharides, including NAOs, AOs, and AHG, have a strong potential for use in pharmaceuticals, cosmetics, food, and bioenergy, as an alternative to overcome the limitations of traditional methods and materials.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflicts of interest.

Ethics approval This article contains studies with experimental rats, which was approved by Institutional Animal Care and Use Committee (permission number of 2013-05-121) at Gyeonggi Bio Center, Republic of Korea.

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