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Rapid and robust enzymatic sensing and quantitation of 3,6-Anhydro-L-galactose in a heterogeneous sugar mixture

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ABSTRACT

3,6-Anhydro-L-galactose (L-AHG) is a rare sugar found in agar polysaccharides. L-AHG has been used as a bioactive compound over the past few years. While the chromatographic or mass-spectrometric quantitation of L-AHG is quite sensitive and accurate, these methods require a reference standard and an intensive sample preparation procedure. We developed an enzymatic assay for rapid and robust quantitation of L-AHG using anhydrogalactose dehydrogenase cloned from *Vibrio* sp. EJY3 (*Vej*AHGD). *Vej*AHGD is a $NADP^+$ - dependent enzyme which catalyzes the oxidation of L-AHG with a stoichiometric ratio of 1:1. Kinetic characterization of the enzyme showed a Km value of 0.19 ± 0.01 mM. The activity of the enzyme was optimum at 20 °C and pH 8.0. The half-life of enzymatic activity was 12 h under optimum condition. *Vej*AHGD was highly specific to L-AHG, such that the reaction was not interfered by a variety of mono- or oligo-sugars in a heterogeneous mixture. Except transition metal ions, other cations or chelating agents did not affect the activity of the enzyme. Detection limit of the assay was 0.2 mM at 340 nm in the spectrophotometry. The assay was so rapid to give the result less than 5 min, requiring neither separation nor pretreatment of samples. We suggest application of the assay for detection and quantitation of L-AHG in commercial products and biosensor development.

1. Introduction

3,6-Anhydro-L-galactose (L-AHG) is a rare hexose sugar commonly found in marine polysaccharides. 3,6-Anhydro-L-galactose along with D-galactose makes neoagarobiose [O-3,6-anhydro-α-L-galactopyranosyl (1,3)-D-galactose] that is the repeating unit of agar [1,2]. L-AHG and agaro-oligosaccharides have been demonstrated for their bioactivity in previous studies. Agaro-oligosaccharides have been studied for its carcinostatic, anti-inflammatory apoptotic induction, anti-oxidant and immune-regulation activity [3–5]. Neoagarobiose can be used as a moisturizer with skin whitening activity [6]. It was proposed that the bioactivity of agaro-oligosaccharides is due to the AHG moiety [5]. The recent studies have proven that the L-AHG has antioxidant and skin whitening properties, which make it a vital active ingredient in cosmetics [7].

The detection and quantitation of L-AHG is essential not only for the study of L-AHG metabolism but also for the quality control of commercial products having L-AHG as an active ingredient. While detection and qualitative analysis of L-AHG can be simply performed using Thin Layer Chromatography (TLC) [7], few methods are available for the quantitative analysis. Quantitative analysis of L-AHG includes specific instrumental analysis such as Liquid Chromatography (LC), Gas Chromatography - Mass Spectrometry (GC-MS) or non-specific spectrophotometric 3,5-dinitrosalicylic (DNS) assay [7–9]. Instrumental analysis is an accurate and precise method for quantitative analysis of L-AHG, but demands a reference standard or chromatographic separation to determine the relative amounts. Furthermore, sample pre-treatment, extraction and derivatization are often pre-requisites before loading into the instrumental devices for analysis. These pre-analysis steps are time-consuming and cost-ineffective to handle large number of samples in a high throughput analysis.

Spectrophotometric DNS assay is cost-effective and saves time but fails to distinguish L-AHG from other reducing sugars. Because the DNS assay gives the non-specific collective amount of reducing sugars in a solution. Therefore, DNS assay is inappropriate for

Abbreviations: L-AHG, 3,6-Anhydro-L-galactose; AHGD, Anhydrogalactose dehydrogenase.

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detection and quantitation of L-AHG in a heterogeneous mixture of reducing sugars. Although these analytical procedures are generally used in many experimental studies, more time- and cost-effective analytical method is preferable for detection and quantitative analysis of L-AHG such as in quality control of commercial products.

In a previous study, we isolated a marine microorganism *Vibrio* sp. strain EJY3 that can utilize L-AHG as the sole carbon source [10]. The catabolic pathway of L-AHG begins with 3,6-anhydro-alpha-L-galactose dehydrogenase (anhydrogalactose dehydrogenase or AHGD) [11]. AHGD is an aldehyde dehydrogenase enzyme (EC 1.2.1.92) belonging to the oxidoreductase family of enzymes. This enzyme catalyzes the oxidation of L-AHG to 3,6-anhydrogalactonate in the presence of $NADP^+$ as the cofactor. During the reaction process, $NADP^+$ is reduced to NADPH in 1:1 stoichiometric ratio with L-AHG. Since NADPH has a characteristic absorbance maximum at 340 nm (Molecular coefficient = 6.22 mM^{-1} cm^{-1}), concentration of NADPH can be determined by spectrophotometric analysis at 340 nm.

Since AHGD is the only known enzyme that is capable of oxidizing L-AHG using $NADP^+$ as cofactor, we adopted the enzymatic reaction of AHGD of *Vibrio* sp. strain EJY3 (*Vej*AHGD) to develop a novel highly specific quantitative assay for L-AHG. There was no enzymatic assay available for the quantitative analysis of L-AHG prior to this study in peer-reviewed literature.

The simple *Vej*AHGD enzymatic assay eliminates the necessity of labor-intensive pre-analysis steps in chromatography or mass spectrometry but quantifies the L-AHG concentration using typical UV spectrophotometry by stoichiometric conversion of NADPH. *Vej*AHGD enzymatic assay combines accuracy and convenience, which makes it ideal for high throughput study and quality control analysis of commercial products favoring simple and quick methods.

In this study, we performed comprehensive molecular characterization and determined the kinetic parameters of *Vej*AHGD. A simple enzymatic assay procedure was developed based on the molecular characterization of the enzymatic activity. The capability of the enzymatic assay was optimized under various conditions in terms of the activity and stability of the enzyme. This is a comprehensive survey on assay parameters of *Vej*AHGD optimized for bio-sensing and quantitative analysis of L-AHG.

2. Results and discussion

2.1. Optimum pH and temperature of VejAHGD

The effect of pH was determined over the range of 3.0–13.0, using the assay described in the method section 4.3. *Vej*AHGD showed the maximum activity at pH 8.0. Enzyme activity was observed over 90% of its maximum within a small pH range from 8.0 to 11.0 (Fig. 1A). At pH values lower than 5, activity of the enzyme was drastically reduced.

Similarly, the effect of temperature on the activity of *Vej*AHGD was determined over the range of 4 °C–75 °C, using the assay described in the method section 4.3. Maximum activity of *Vej*AHGD was observed at 20 °C and the activity was drastically decreased with increasing temperature (Fig. 1B).

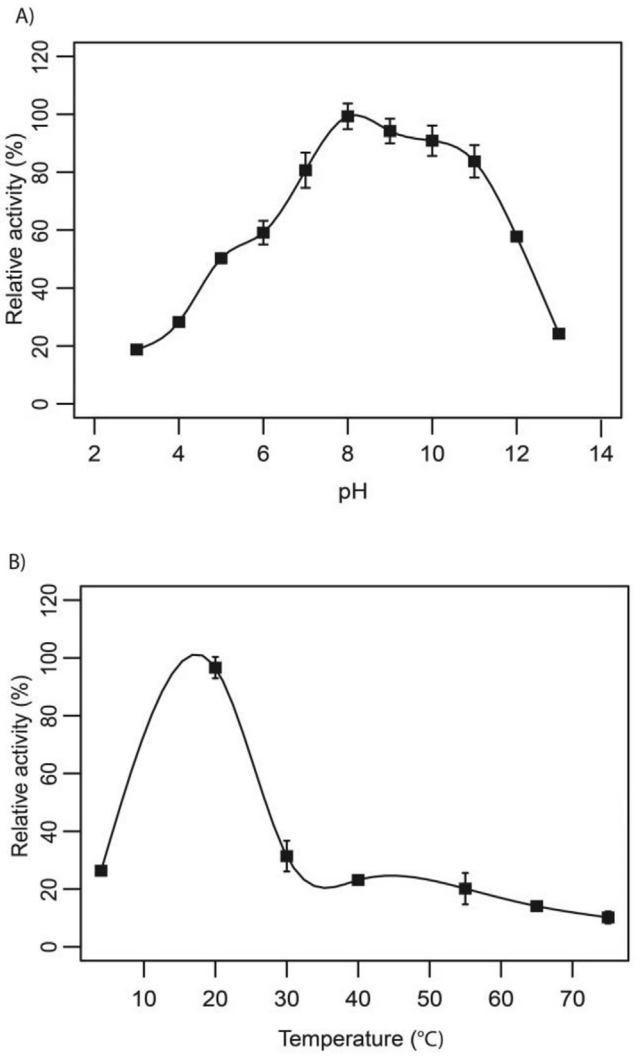

Fig. 1. Effect of temperature and pH over the activity of *Vej*AHGD. A). Effect of pH over the activity of *Vej*AHGD. B). Effect of temperature over the activity of *Vej*AHGD. Activity of the *Vej*AHGD was measured at temperatures ranging from 4 °C to 75 °C and at pH ranging from 3 to 13 in the presence of 1 mM L-AHG and 5 mM $NADP^+$.

2.2. pH stability and thermostability of VejAHGD

To check the pH stability of *Vej*AHGD, pure enzyme was incubated in buffers having pH values ranging from 4 to 13, while keeping the temperature at a constant. Enzyme was incubated up to 12 h at 0 °C and the residual activity of the enzyme was determined at different time points. As shown in Fig. 2A, stability of the *Vej*AHGD was found to be maximum at slightly alkaline pH, within the range of pH 8–10. Whereas at pH below 5, stability of the enzyme was drastically reduced. Coagulation of the enzyme was observed at pH below 5. These suggest that slight alkaline pH (pH 8–10) is the most favorable condition for *Vej*AHGD for its function and the storage.

To check the thermostability of *Vej*AHGD, pure enzyme was incubated at different temperatures ranging from 0 to 60 °C, while keeping the pH of the medium at a constant. Incubation period was up to 12 h at 20 mM Tris buffer (pH 8.0). The residual activity was measured after the completion of incubation period. *Vej*AHGD showed strong temperature sensitivity. As shown in Fig. 2B, residual activity of the enzyme was decreased with increasing temperature. At 0 °C, *Vej*AHGD showed its maximum stability by maintaining almost a constant residual activity without decreasing. At the optimum temperature range for enzyme activity (20–30 °C), enzyme showed a considerably high stability. Half-life of the enzyme at 20 °C was determined as 12 h ($t_{½}$ at 20 °C = 12 h). Whereas, at 50 °C half-life of the enzyme is less than 10 min ($t_{½}$ at 50 °C < 10 min), suggesting that it is highly unstable at temperatures above 50 °C.

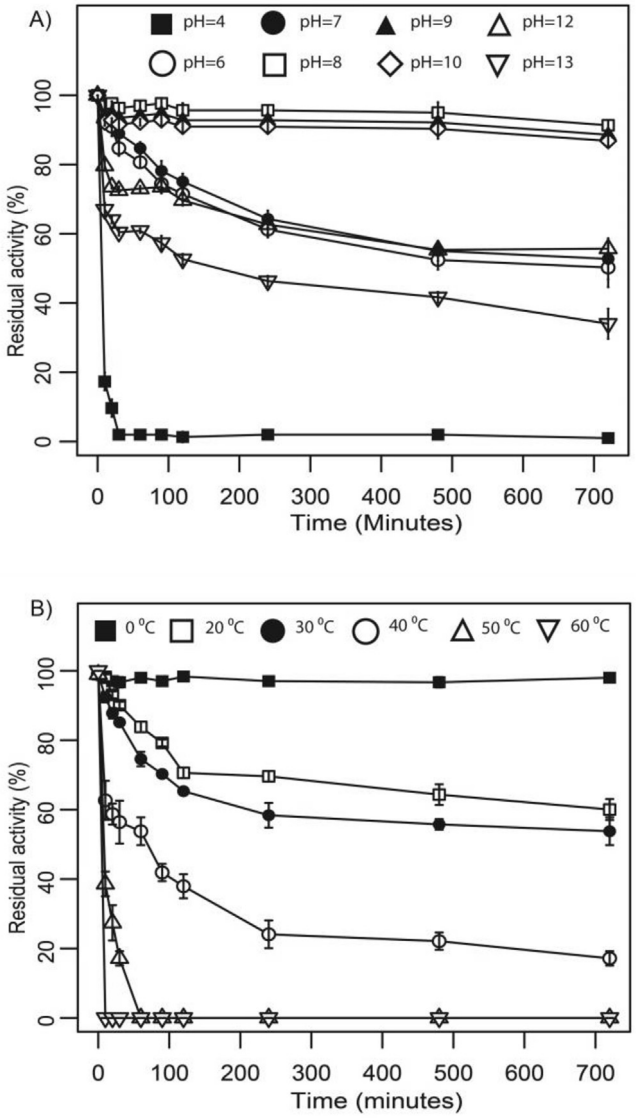

Fig. 2. pH and thermostability of *Vej*AHGD. A). Effect of temperature over the stability of *Vej*AHGD. Enzyme was incubated at different temperatures at constant pH and the residual activity of *Vej*AHGD was determined at different time points. B). Effect of pH over the stability of *Vej*AHGD. Enzyme was incubated at different pH at constant temperature and the Residual activity of *Vej*AHGD was determined at different time points.

2.3. Kinetic parameters of VejAHGD

Kinetic parameters of *Vej*AHGD was determined using L-AHG as the substrate and $NADP^+$ as the cofactor. The K_m and V_{max} were determined as 0.19 ± 0.01 mM (SD, n = 3) and 0.36 ± 0.01 mg/min (SD, n = 3) respectively. Catalytic efficiency (K_{cat}) of *Vej*AHGD using L-AHG was determined to be 0.357 ± 0.014 min^{-1} (SD, n = 3) and K_{cat}/K_m was found to be 1.91 ± 0.05 mM^{-1} min^{-1} (SD, n = 3) (Supplementary Fig. S1 and Supplementary Table S1).

2.4. Substrate specificity of VejAHGD

Substrate specificity of the *Vej*AHGD enzyme was examined for various mono-, di- and oligosaccharide substrates. Apart from the natural substrate of *Vej*AHGD, which is L-AHG, several other sugar substrates were tested to determine the substrate specificity of *Vej*AHGD. These include the optical isomer of natural substrate, 3,6-Anhydro-D-galactose; some commonly found hexose sugars such as D-glucose, D-galactose, D-fructose; disaccharides such as sucrose and maltose; pentose sugars including arabinose and xylose. Agar derived substrates such as agaro-oligosaccharides (agarohexose and agarotetrose) and neoagarobiose were also tested as substrates. Interestingly, none of these substrates, other than L-AHG, was used as a substrate by *Vej*AHGD. These results indicate that *Vej*AHGD is highly specific for L-AHG.

2.5. Effect of other sugars over 3,6-Anhydro-L-galactose

L-AHG is often found in a heterogeneous mixture of sugars. Therefore, it is necessary to test the interfering effects of other sugars. To check whether other sugar substrates can exert a competitive inhibition effect by binding to the active site of *Vej*AHGD, activity of *Vej*AHGD was measured in a heterogeneous sugar mixture. Hexose sugars, pentose sugars and agar derived oligosaccharide substrates were used at 1 mM concentration along with L-AHG. In addition, the effect of product mimics such as D-glucuronic acid and D-galacturonic acid over L-AHG was also assessed at 1 mM concentration. Even with the presence of other sugars at high concentration in the mixture, only a negligible interference was exerted by those sugars. Pure L-AHG and the mixture both showed the same kinetic behavior as shown in Fig. 3A and B. Competitive or noncompetitive inhibition profiles were not observed when L-AHG was used in a heterogeneous mixture of sugars. Further, we calculate the V_{max} and K_M values for each heterogeneous sugar mixture as the substrate and compared those values with L-AHG as the pure substrate (Table 1). Considering the experimental errors associated with measurements, V_{max} values for heterogeneous sugar mixture were within ±7.5% range of the V_{max} value for pure L-AHG substrate. Similarly, K_M Values for heterogeneous sugar mixture were also within ±7.5% range of the K_M value for pure L-AHG substrate. These indicated that other sugar compounds in heterogeneous mixture does not exert any competitive or non-competitive effect over the activity of *Vej*AHGD.

2.6. Effect of salts

Effect of various salts over the activity of *Vej*AHGD was tested. Commonly found monovalent, divalent cations and transition metal ions were selected to determine their effect over the activity of *Vej*AHGD (Table 2). Final concentration of the ion was kept at 1 mM. Monovalent cations, Na^+ and K^+ had no effect on the activity of *Vej*AHGD. In case of divalent cations, Mg^{2+} had no effect on activity whereas Ca^{2+} reduced the activity by 20%. Among the transition metal ions, Mn^{2+} reduced the activity by 30%, whereas Co^{2+}, Ni^+, Cu^{2+}, Zn^{2+} and Cd^{2+} completely inhibited the enzyme. Further, effect of chelating agents was tested using 1 mM EDTA and found to have no effect on enzyme activity.

The enzyme can be easily inactivated by transition metals. We tested for five transition metals and all of them permanently inactivated the enzyme. However, other commonly found cations such as Na^+, K^+ and Mg^{2+} or metal chelating agents had no effect on the enzyme activity. Therefore, we suggest to use 1 mM EDTA, if the mixture is suspected to contain transition metals.

2.7. VejAHGD enzymatic assay

Since agar is the major source of L-AHG, it is often found in a mixture of D-galactose and agaro-oligosaccharides such as agarotetrose and agarohexose. Further, cosmetics which utilize L-AHG as the active ingredient may contain many other interfering materials. Therefore, any analytical method which is going to be used

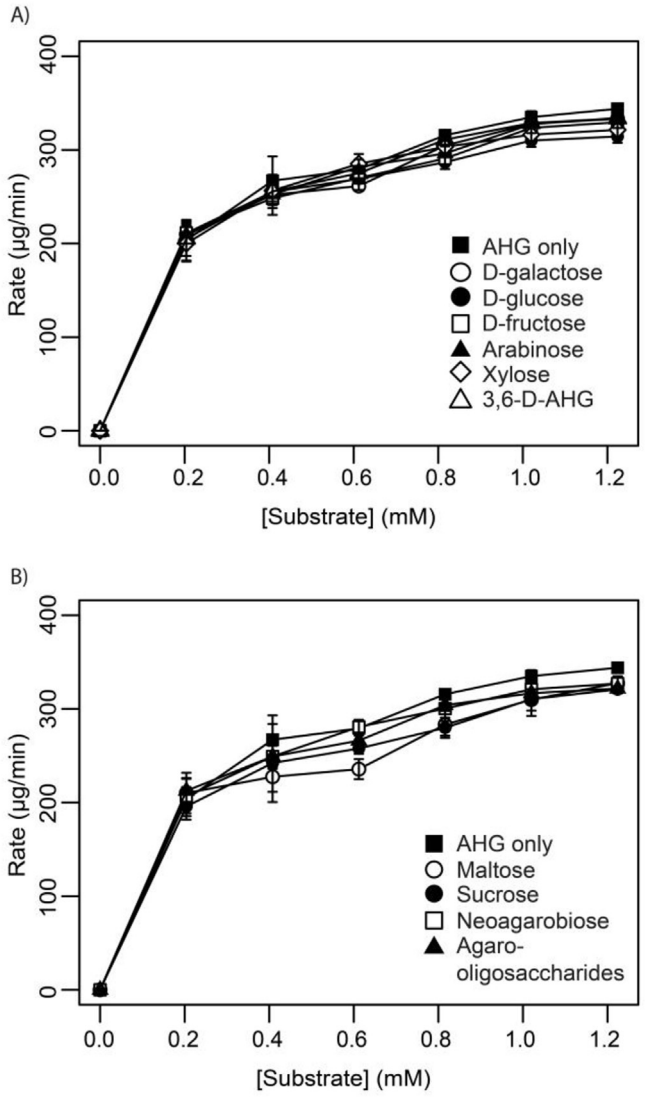

Fig. 3. Effect of other sugars over the activity of *Vej*AHGD in a heterogeneous mixture. A). Effect of Other C_6 and C_5 sugars over the activity of *Vej*AHGD. B). Effect of disaccharides and oligosaccharides over the activity of *Vej*AHGD. Activity of *Vej*AHGD was determined in the presence of 1 mM L-AHG and 1 mM of other sugars.

Table 1
Comparison of V_{max} and K_M values of pure L-AHG and L-AHG in heterogeneous sugar mixture as the substrate for *Vej*AHGD. Other sugars in heterogeneous mixture were used in 1 mM concentration.

Composition	V_{max} (mg/min) Value ± SD (n = 3)	K_M (mM) Value ± SD (n = 3)
L-AHG only	0.36 ± 0.01	0.19 ± 0.01
L-AHG + D-glucose	0.37 ± 0.01	0.18 ± 0.01
L-AHG + D-galactose	0.34 ± 0.01	0.18 ± 0.01
L-AHG + D-fructose	0.34 ± 0.02	0.17 ± 0.01
L-AHG + Arabinose	0.38 ± 0.01	0.19 ± 0.01
L-AHG + Xylose	0.37 ± 0.01	0.19 ± 0.01
L-AHG + 3,6-D-AHG	0.37 ± 0.01	0.19 ± 0.01
L-AHG + Maltose	0.33 ± 0.02	0.18 ± 0.01
L-AHG + Sucrose	0.35 ± 0.01	0.19 ± 0.01
L-AHG + D-galacturonic acid	0.35 ± 0.01	0.18 ± 0.01
L-AHG + D+glucuronic acid	0.36 ± 0.01	0.18 ± 0.01
L-AHG + Neoagarobiose	0.37 ± 0.01	0.19 ± 0.01
L-AHG + Agaro-oligosaccharides	0.37 ± 0.01	0.18 ± 0.01

Table 2
Relative activity of *Vej*AHGD in the presence of various metal ions and chelating agents. Activity of pure *Vej*AHGD was considered as 100% activity.

Category	Metal ion/Chelating Agent	Relative Activity (%)
Without metal ions		100%
Mono valent cations	Na^+	109.1 ± 1.3%
	K^+	101.7 ± 1.5%
Divalent cations	Mg^{2+}	96.3 ± 0.8%
	Ca^{2+}	80.8 ± 1.1%
Transition metals	Mn^{2+}	68.2 ± 4.6%
	Co^{2+}	Complete inhibition
	Ni^+	Complete inhibition
	Cu^{2+}	Complete inhibition
	Zn^{2+}	Complete inhibition
	Cd^{2+}	Complete inhibition
Chelating agents	EDTA	132.2 ± 3.9%

for quantitation of L-AHG should be highly specific and minimally interfered by other chemical compounds. In addition, it should meet the demands of high throughput analysis such as low detection limit, low sample input and quick assay time.

*Vej*AHGD is ideal for developing an enzymatic quantitative assay for L-AHG. It showed a high specificity for L-AHG than other commonly found monosaccharides, disaccharides and agaro-oligosaccharides (Refer to section 2.4). Further, these substrates did not act as inhibitors to the enzyme activity by competitively binding to the active site (Refer to section 2.5). Due to the substrate specificity of *Vej*AHGD enzyme, the amount of L-AHG can be accurately determined regardless of the composition of the mixture. Even though L-AHG is commonly found as a saccharification product of agar, this method is not limited to quantify L-AHG in saccharification mixture. It can be extended to quantify L-AHG in any heterogeneous mixture such as cosmetics and pharmaceuticals.

*Vej*AHGD enzymatic assay has several beneficial features. It has combined the constructive features of both DNS assay and LC or GC/MS. Unlike DNS assay, exact amount of L-AHG can be determined accurately. Unlike GC/MS or LC, the assay requires no sample preparation before the quantification. We have optimized the method to be used in the 96 well plate for high throughput sample analysis.

The assay can be utilized for microscale analysis of L-AHG. It is sensitive enough to detect low concentrations of L-AHG with a minimum detection limit of 0.2 mM. Since a spectrophotometric method was used in this assay, the maximum limit is restricted to 1 mM. In addition, assay involves few mixing steps and the reaction can be completed within 5 min. The optimum temperature of this assay, which is 20 °C, allows the reaction to be performed at room temperature. And the enzyme is compatible with commonly found buffer, such as Tris buffer at pH 8.0.

To check the accuracy of our method under optimized assay conditions, we used a GC-MS quantified pure L-AHG samples having different concentrations and quantify the amount of L-AHG using *Vej*AHGD assay. As shown in Fig. 4A, L-AHG concentrations obtained by *Vej*AHGD assay were in the range of the concentrations obtained by GC-MS. This proved that accuracy of *Vej*AHGD assay was similar to that of GC-MS, whereas the convenience of the method is far superior to GC-MS.

To assess the quantitative linearity of the *Vej*AHGD assay, L-AHG standard (quantified by GC-MS) was used. L-AHG was used at different concentrations from 0.1 mM to 1 mM in the *Vej*AHGD assay, and the corresponding absorbance was measured at 340 nm. Response was plotted against the L-AHG concentration used (Fig. 4B). Within the concentration range of 0.1 mM–1.0 mM, *Vej*AHGD assay showed a linear response against the L-AHG concentration. *Vej*AHGD assay is highly reproducible under optimum

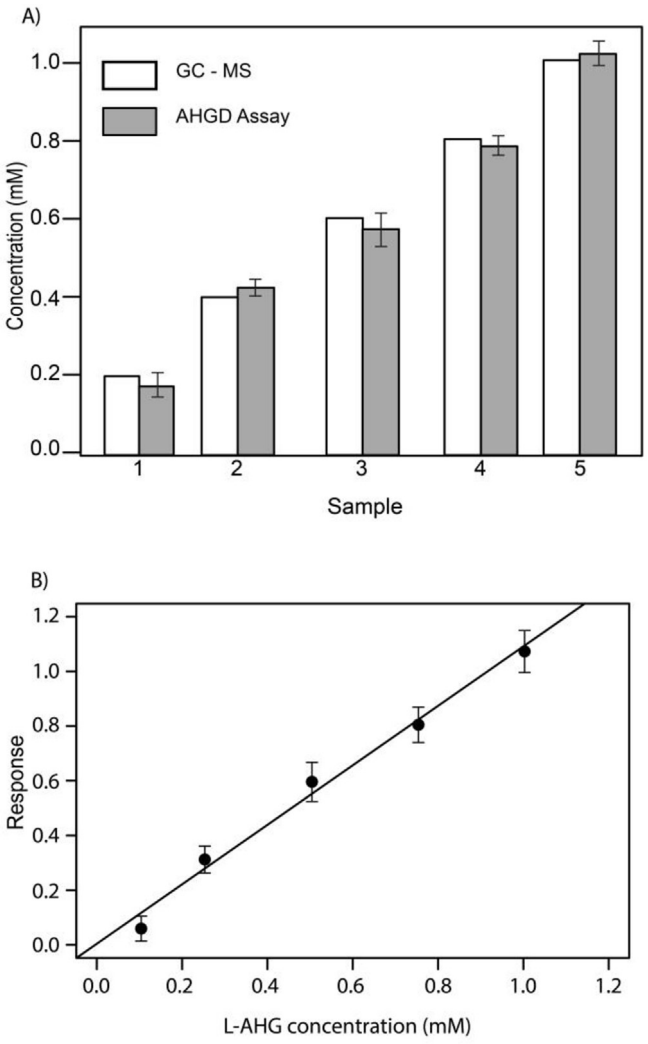

Fig. 4. Accuracy and linearity of *Vej*AHGD assay. A). Accuracy of *Vej*AHGD assay. L-AHG concentration of 5 different samples were obtained by GC-MS and their concentration were determined by *Vej*AHGD assay, keeping the respective GC-MS obtained concentration as a reference. B). Linearity of *Vej*AHGD assay. Standardized L-AHG samples of L-AHG at different concentrations from 0.1 mM −1 mM were used in *Vej*AHGD assay. Response was plotted against the L-AHG concentration.

conditions. Each data point obtained was triplicated and presented as mean ± standard deviation.

*Vej*AHGD enzymatic assay was developed by eliminating the cons of and combining the pros of currently available L-AHG quantitation methods. Combined with the robustness and convenience, *Vej*AHGD assay can be suggested as the ideal method for the quantitative analysis of L-AHG in a heterogeneous mixture.

3. Conclusion

Anhydrogalactose dehydrogenase assay is highly specific for L-AHG and minimally interfered by other sugars including its stereoisomer in a heterogeneous mixture. Stability was enough to maintain constant level of activity through the incubation period of the assay. Enzymatic oxidation of L-AHG to 3,6-anhydrogalactonate simultaneously reduces $NADP^+$ to NADPH in 1:1 molar ratio which can be measured spectrophotometrically at 340 nm. Therefore, AHGD assay is robust and the most convenient analytical method for quantitative analysis of L-AHG in a heterogeneous mixture.

4. Experimental

4.1. Cloning and expression of enzymes

Vibrio sp. EJY3 was grown in in minimal broth containing 2.3% (w/v) aquarium sea salt mix (Instant Ocean Sea Salts; Aquarium Systems, Mentor, OH, USA), 0.1% (w/v) yeast extract, 0.05% (w/v) NH4Cl and 10 mM Tris-HCl buffer (pH 7.4). Genomic DNA was isolated using a commercial genomic DNA extraction kit (Bioneer, Daejeon, Korea). The gene encoding for Anhydrogalactose dehydrogenase (*Vejahgd*, NCBI gene ID: 11666893) was amplified using genomic DNA of *Vibrio* sp. strain EJY3 as the template and the primers listed in Supplementary Table S2. Signal peptide of the protein was removed to improve the solubility of the overexpressed protein in cytosol. Amplified gene was cloned in to a pBAD/myc-His vector with six histidine tag at carboxyl terminus by Ligation Independent Cloning (LIC) method. Cloned plasmids were transformed in to *E. coli* BW25113 for overexpression. The strains and plasmids are listed in Supplementary Table S3. Cells were grown in Luria-Bertani medium (LB; BD, Sparks, MD, USA) containing 100 μg/ml Ampicillin (Duchefa Biochemie, Haarlem, Netherlands) until the cells reach to log phase. Overexpression of the recombinant protein was induced by adding 20% (w/v) Arabinose (Sigma-Aldrich, St. Louis, MO, USA) up to a final concentration of 0.1% (w/v). Cells were incubated at 16 °C for 12 h in 180 rpm shaking platform [11].

4.2. Extraction and purification of recombinant enzyme

Cells were harvested by centrifugation and resuspended in lysis buffer containing 20 mM Tris-Cl (pH 8.0), 200 mM Sodium Chloride, 5 μM β-mercaptoethanol and 5% Glycerol. Cells were disrupted by sonication under low temperature and soluble and insoluble fractions were separated by centrifugation at 3500 rpm at 4 °C for 30 min. Recombinant protein *Vej*AHGD was purified using a His-Trap column (GE Healthcare, USA). Purified protein was qualitatively analyzed by SDS-Polyacrylamide Gel Electrophoresis. The amount of total protein in the crude extract was determined by Bradford protein assay (Bio-Rad, Hercules, CA, USA) using bovine serum albumin (BSA) (Takara, CA, USA) as the standard [11].

4.3. AHGD enzymatic assay

For the *Vej*AHGD enzymatic assay, 30 μg of pure recombinant enzyme was incubated in 20 mM Tris buffer (pH 9.0) containing 0.5 mM L-AHG and 2.5 mM $NADP^+$ at 20 °C. Reaction was performed for 30 min and the concentration of NADPH was determined by spectrophotometry at 340 nm (Molar extinction coefficient = 6.22 mM^{-1} cm^{-1}). One unit of *Vej*AHGD enzyme was defined as the microgram of L-AHG oxidized by the enzyme per minute at 20 °C and pH 8.0.

4.4. Quantitative analysis of L-AHG by GC–MS

L-AHG is commercially unavailable. Therefore, D-form of 3,6-anhydrogalactose (D-AHG; Dextra Laboratories, Berkshire, UK) was used as the standard. D-AHG was dissolved in 50 mM Tris-HCl buffer (pH 7.4). After the standards were aliquoted into 1.5 ml tubes they were dried in a centrifugal vacuum concentrator at 25 °C for 6 h. The dried sugar samples were methoximized and trimethylsilylated prior to GC-MS analysis. The carbonyl groups of the sugars were protected by methoximation using 50 μl of 2% (w/v) methoxyamine hydrochloride in pyridine at 75 °C for 30 min. For trimethylsilylation of the sugars, 80 μl of N-methyl-N-trimethylsilyltrifluoroacetamide (MSTFA; Fluka, St Louis, MO, USA) were subsequently added to methoxymized samples at 45 °C for 30 min.

One microliter of the sample was injected in split injection mode with a split ratio of 9.6 into an Agilent 7890A GC/5975C MSD system (Agilent Technologies, Wilmington, DE, USA) equipped with a DB-5MS capillary column (30 m × 0.25 mm, 0.25 μm film thickness, Agilent Technologies). The oven temperature of the GC was initially set at 100 °C for 3.5 min, after which the temperature was increased to 160 °C at 15 °C/min, where it was held for 20 min, then increased to 200 °C at 20 °C/min, where it was held for 15 min, and finally increased to 280 °C at 20 °C/min, where it was held for 5min. The mass spectra were recorded in a scan range 50–600*m/z* at 70 eV of electron impact, and the temperature of the ion source of MS was 280 °C. Finally, the amounts of L-AHG were calibrated based on the peaks of each compound in the total ion chromatogram [12].

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.carres.2017.04.022.

References

[1] C.H. Araki, Bull. Chem. Soc. Jpn. 29 (1956) 543–544.
[2] D. Renn, Trends Biotechnol. 15 (1997) 9–14.
[3] H. Chen, X. Yan, P. Zhu, J. Lin, Nutr. J. 5 (2006) 1–12.
[4] H.M. Chen, L. Zheng, X.J. Yan, Food Technol. Biotechnol. 43 (2005) 29–36.
[5] J.Tomono, H.Sagawa, I.Kato, US patent 7622291 B2 (2009).
[6] R. Kobayashi, M. Takisada, T. Suzuki, K. Kirimura, S. Usami, Biosci. Biotechnol. Biochem. 61 (1997) 162–163.
[7] E.J. Yun, S. Lee, J.H. Kim, B.B. Kim, H.T. Kim, S.H. Lee, J.G. Pelton, N.J. Kang, I.G. Choi, K.H. Kim, Appl. Microbiol. Biotechnol. 97 (2013) 2961–2970.
[8] H.T. Kim, S. Lee, K.H. Kim, I.G. Choi, Bioresour. Technol. 107 (2012) 301–306.
[9] G. Miller, Anal. Chem. 31 (1959) 426–428.
[10] H. Roh, E.J. Yun, S. Lee, H.J. Ko, S. Kim, B.Y. Kim, H. Song, K. Lim, K.H. Kim, I.G. Choi, J. Bacteriol. 194 (2012) 2773–2774.
[11] E.J. Yun, S. Lee, H.T. Kim, J.G. Pelton, S. Kim, H.J. Ko, I.G. Choi, K.H. Kim, Environ. Microbiol. 17 (2015) 1677–1688.
[12] E.J. Yun, M.H. Shin, J.J. Yoon, Y.J. Kim, I.G. Choi, K.H. Kim, Process Biochem. 46 (2011) 88–93.