Pre-print manuscript of following article with supplementary material.

Tetrahedron 2015, 71 (9), p1419-1424

DOI: 10.1016/j.tet.2015.01.019; <http://dx.doi.org/10.1016/j.tet.2015.01.019>

Graphical Abstract

Selective Purification of Intestinal Maltase Complex by Affinity Chromatography Employing an Uncompetitive Inhibitor as the Ligand

Eisuke Kato,* Hiroki Tsuji and Jun Kawabata*

Laboratory of Food Biochemistry, Division of Applied Bioscience, Graduate School of Agriculture, Hokkaido University, Kita-ku, Sapporo, Hokkaido 060-8589, Japan

*Corresponding Authors

E. Kato: eikato@chem.agr.hokudai.ac.jp; Tel/Fax: +81-11-706-2496

J. Kawabata: junk@chem.agr.hokudai.ac.jp; Tel/Fax: +81-11-706-2496

Abstract

Affinity chromatography of specific enzymes is limited by the presence of related enzymes and the limited selectivity of the affinity ligand. We synthesized and investigated the use of an uncompetitive inhibitor as an affinity ligand, to leverage its three-component interactions. Use of the potent α -glucosidase uncompetitive inhibitor 2-aminoresorcinol as the ligand of the affinity gel offered selective purification of maltase-glucoamylase complex from the crude mixture of intestinal α -glucosidases.

Keywords

Affinity chromatography, uncompetitive inhibitor, glucosidase inhibitor, intestinal glucosidase, maltase-glucoamylase complex

1. Introduction

Affinity chromatography is a powerful tool for the purification of target molecules.^{1,2} The technique makes use of the specific interactions between pairs of molecules. Expansion of protein expression techniques and development of a method to design and create genetically engineered protein have enabled use of affinity chromatography as an easy and efficient way to purify expressed proteins. Tag sequences, for example the His- and FLAG-tags, offer an excellent way to distinguish engineered proteins from native proteins.^{3,4} The use of synthetic affinity gels that incorporate a designed ligand offers a systematic approach for purification of tagged proteins.^{5,6}

In contrast to engineered proteins, affinity purification of native proteins remains a challenging task. In particular, purification of a specific enzyme from the target organism is often complicated by the presence of related enzymes that catalyze the same reaction but target different substrates. Competitive or mixed-mode inhibitors are frequently selected as the affinity gel ligand for the purification of enzymes.⁷⁻¹⁰ However, these inhibitors often possess inhibitory activity toward multiple related enzymes, making selective adsorption of the target enzyme to the affinity gel difficult when these inhibitors are used as a ligand.^{11,12} Furthermore, when highly active inhibitors are used, desorption of the target enzyme from the gel requires severe conditions and may co-elute proteins adsorbed by non-specific interactions, hampering purification.

Uncompetitive inhibitors are characteristic inhibitors that bind only to enzyme-substrate complexes. This interaction makes uncompetitive inhibitors more selective and efficient affinity ligand candidates. Because uncompetitive inhibitors bind only to the enzyme-substrate complex, an appropriate substrate is required for them to inhibit enzyme activity. In turn, the substrate can increase the selectivity of the inhibitor. Thus, even if an uncompetitive inhibitor has low selectivity between several enzymes, the presence of the specific substrate is expected to increase the selectivity of the inhibitor.

Inhibitory activity	
	IC ₅₀ (μM)	
	maltase	4.4
2-aminoresorcinol (1)	sucrase	2.4

Figure 1. Structure and α -glucosidase inhibitory activity of 2-aminoresorcinol (1).

2-Aminoresorcinol (1) is an uncompetitive α -glucosidase inhibitor that has high

inhibitory activity toward intestinal glucosidases (Figure 1),¹³ which contain a mixture of two glucosidase complexes, maltase-glucoamylase (MG) and sucrase-isomaltase (SI). Inhibitors of intestinal glucosidases are effective for the treatment of diabetes mellitus and have been extensively studied.¹⁴⁻¹⁶ Although separation of these complexes has been reported,^{17,18} the steps required are complicated, and most studies have used a mixture of MG and SI, providing rather unclear results concerning the target enzyme of the inhibitors. Thus, a one-step method for purifying these enzymes would facilitate this line of research.

Here, we show for the first time, to the best of our knowledge, the efficacy of an uncompetitive inhibitor as an affinity gel ligand. Using 2-aminoresorcinol (**1**) as the affinity ligand, we separated intestinal MG from rat intestinal crude enzyme mixture using only simple techniques.

2. Results and discussion

2.1. Synthesis of affinity ligand

Based on the structure–activity relationship study of (**1**),¹⁹ an affinity ligand (**2**) was designed and synthesized (Scheme 1). The 2-Nitroresorcinol derivative (**3**) was hydrogenated in the presence of di-*tert*-butyl dicarbonate to yield (**4**). Initial trial for the amide bond formation reaction between **4** and ethylenediamine linker gave low yield (~10%). In addition, the isolation of the resulting product required repeated separations by silica-gel column due to contaminations of unreacted **4**. This was reduced by the protection of *para*-hydroxyl group with methoxymethyl group which also gave higher yield for the amide bond formation reaction. Thus, *para*-hydroxyl group of **4** was protected employing equivalent amount of methoxymethyl chloride with diisopropylethylamine as a base at rt. Due to hydrogen bond between carbonyl group, *ortho*-hydroxyl group has lower reactivity compared to *para*-hydroxyl group and **5** was selectively obtained with slight amount of bis-hydroxyl group protected product, and no *ortho*-hydroxyl group protected product was obtained. Then, the methyl ester was hydrolyzed to yield (**6**). A short linker, *N*-Cbz-ethylenediamine, was reacted with (**6**) in the presence of EDCI and then the Boc group was removed by hydrogen chloride to yield intermediate (**8a**). Deprotection of the amino group at the end of the linker yielded the desired ligand (**2**).

An affinity gel (**9**) was synthesized by the reaction of ligand (**2**) with NHS-activated sepharose 4 FF (GE Healthcare Co.) under the conditions recommended by the manufacturer (Scheme 1). The amount of residual ligand (**2**) or released NHS in the coupling solution was monitored by high performance liquid chromatography (HPLC)

and the ligand concentration or coupling efficiency was calculated. Approximately 19.8 $\mu\text{mol/mL}$ gel, or 95% of the activated residue on the gel, was estimated to be modified by the ligand (**2**).

Scheme 1. Synthesis of the affinity ligand. (a) H_2 , Pd/C, Boc_2O , THF. (b) MOMCl, DIPEA, DMF. (c) NaOH, MeOH, H_2O . (d) for **7a**: *N*-Cbz-ethylenediamine HCl, EDCI, DIPEA, DMAP, DMF. for **7b**: *N*-Acetylenediamine, EDCI, DIPEA, DMAP, DMF. (e) 4 M HCl/dioxane, 0°C . (f) H_2 , Pd/C, MeOH, 0°C . (g) NHS-activated sepharose FF, 0.2 M NaHCO_3 , 0.5 M NaCl aq.

2.2. Uncompetitive mode of inhibition of affinity ligand

To confirm the uncompetitive mode of inhibition of the derived ligand (**2**), *N*-acetylated ligand (**8b**) was prepared and subjected to glucosidase inhibition assay. Variation of ligand (**8b**) concentration depicted parallel lines in Lineweaver-Burk plot for both maltase and sucrase (Figure 2). The curve is representative for the uncompetitive inhibitor and the result certified that the derived ligand possesses uncompetitive mode of inhibition. The inhibition constant was estimated to be $K_i = 10.6 \mu\text{M}$ for maltase and $K_i = 14.9 \mu\text{M}$ for sucrase from Michaelis-Menten equation (Supplementary Fig. 1). Given the satisfying result of the ligand, we then synthesized the affinity gel (**9**).

Figure 2. Lineweaver-Burk plot of maltase (A) and sucrase (B) inhibition by the acetylated ligand (**8b**). Each line indicates varied concentration of **8b**.

2.3. Purification of MG complex

To test the uncompetitive mode of adsorption, crude enzyme prepared from rat intestinal acetone powder, containing both MG and SI, was passed through the column filled with affinity gel (**9**) with or without maltose, and the adsorbed maltase was eluted with a sodium chloride solution. As shown in Table 1, the maltase activity of the eluted fractions appeared only when maltose was added to the buffer. This clearly indicated that the ligand was able to adsorb the target enzyme when the substrate was added,

reflecting the inhibitory mechanism of the uncompetitive inhibitor. Furthermore, the result suggests that the adsorbed enzyme can be eluted simply by removing the substrate, instead of by adding a free inhibitor, changing the pH, or using high concentrations of salt, methods which are often employed.

Table 1. Uncompetitive mode of adsorption of maltase by affinity gel (9)^a

Condition	Maltose ^b	Adsorbed protein (mg)	Maltase activity	
			Units	U/mg protein
1	+	0.0284	0.08	2.85
2	-	0.0196	< 0.01	-
CE	-	3.37	0.28	0.08

^aCrude enzyme (CE) was passed through the affinity gel (9) with or without maltose and then eluted with 1 M sodium chloride. ^bFollowing buffer was used during adsorption step. +: 3.5 mM maltose, 5 mM EDTA, 0.1 M phosphate buffer (pH7.0). -: 5 mM EDTA, 0.1 M phosphate buffer (pH7.0).

Next, affinity purification of maltase from the crude enzyme was performed. Crude enzyme was passed through the column filled with gel 9 in the presence of maltose. Maltose was continuously added to the buffer during washing step, and was removed from the buffer at the initial elution step. Then, a sodium chloride containing buffer was used for the secondary elution step. The eluate was fractionated and the maltase /sucrase hydrolyzing activity of each fraction was determined (Figure 3). As expected, the fraction eluted by the maltose-free buffer yielded the highest maltase activity (Figure 2, E2). The maltase activity of the purified fraction was up to 78-fold greater than that of the crude enzyme, whereas the sucrase activity was far lower, indicating that MG was selectively purified (Table 2). The remaining sucrase activity in E2 fraction might be due to the interaction with the Sepharose gel, which is reported to possess a weak interaction with Isomaltase.²⁰ Recovery yield of maltase activity in Fr. E2 was 17% among the applied crude enzyme. The low recovery yield is probably due to excess enzyme loaded to the column speculated from the result that 53% of the maltase activity eluted in Fr. W1 and W2.

Figure 3. α -Glucosidase activity of fractions eluted from the affinity column. Maltase activity and sucrase activity designate the sucrose and maltose hydrolyzing activity. The following buffers were used for the Wash (W) and Elute (E) fractions. W1 and W2: 3.5 mM maltose, 5 mM EDTA, 0.1 M sodium phosphate buffer (pH 7.0); E1 and E2: 5 mM EDTA, 0.1 M sodium phosphate buffer (pH 7.0); E3 and E4: 1 M sodium chloride, 5 mM EDTA, 0.1 M sodium phosphate buffer (pH 7.0).

Table 2. α -Glucosidase activity of the crude and purified fractions.^a

Fr.	Maltase		Sucrase		Fold	
	Units	U/mg protein	Units	U/mg protein	Maltase	Sucrase
CE	0.573	0.057	0.917	0.091	1	1
E2	0.100	4.460	0.006	0.254	78	3

^aRepresentative value of three independent experiments.

To confirm the obtained enzyme, fraction E2 was analyzed by native-PAGE, SDS-PAGE, and western blotting (Figure 4). Native-PAGE analysis yielded a single band (Figure 4A), which showed maltase activity (data not shown). SDS-PAGE analysis of the fraction confirmed bands at molecular weights of 130 and 240 kDa, and another two bands above 250 kDa (Figure 4B). The MG complex is reported to consist of five fragments with molecular weights of 134, 245, 350, 410, and 480 kDa, consistent with the current analysis.^{21,17} Western blotting analysis using polyclonal anti-MG antibody showed that all four bands originated from MG (Figure 4C). Thus, all of the purification analyses showed that the purified fraction contained MG as the major component, and that MG was successfully purified using affinity gel (9).

Figure 4. Analysis of the E2 fraction. M: Marker protein, CE: Crude enzyme, E2: E2 fraction. A) Native-PAGE analysis, silver stain. B) SDS-PAGE analysis, silver stain. C) Western blotting analysis with polyclonal anti-maltase-glucoamylase antibody (Santa Cruz Biotechnology, Inc.).

3. Conclusion

In conclusion, we employed an uncompetitive α -glucosidase inhibitor (**1**) as a tool for affinity purification of MG from crude enzyme extract. The unique characteristics of the uncompetitive inhibitor allowed the substrate to act as an additional factor to increase the selectivity of the affinity chromatography, and simultaneously provided a simple and efficient elution procedure. The adsorption and elution steps are the two most important steps in affinity chromatography, and we consider the use of an uncompetitive inhibitor as one of an efficient method for enzyme purification.

4. Experimental methods

4.1. General

Intestinal acetone powder from rat was purchased from Sigma-Aldrich Co. All other commercially available chemicals were purchased from Wako Pure Chem. Ind. Ltd. and used without further purification. Structures of the synthetic compounds were determined by NMR and Mass

spectrometry. Bruker AMX500 or Jeol JNM-EX 270 was used to obtain NMR spectrum and either tetramethylsilane (TMS) or residual solvent peak was used as an internal standard (^1H NMR: TMS 0.00 ppm for CDCl_3 , CD_3OD 3.30 ppm, $\text{DMSO}-d_6$ 2.49 ppm; ^{13}C NMR: CDCl_3 77.0 ppm, CD_3OD 49.0 ppm, $\text{DMSO}-d_6$ 39.5 ppm). Jeol JMS-T100GCV (FD-MS) or Waters LCT Premier (ESI-MS) was used to obtain mass spectrum. IR was measured by FT/IR-4100ST (JASCO Co.). Melting points are measured by MP-S3 (Yanaco New Science Inc.). Absorbance was measured by SynergyTM MX (Bio-tech Instruments Inc.) microplate reader.

4.2. Synthesis

Methyl 3-(*tert*-butoxycarbonylamino)-2,4-dihydroxybenzoate (4).

Compound **3** (220 mg, 1.03 mmol) was dissolved in THF (10 mL) and Pd-C (50 mg) was added. To this mixture, Boc_2O (338 mg, 1.55 mmol) was added and reacted for one day under hydrogen atmosphere. The reaction mixture was passed through the celite pad and evaporated. The residue was purified by silica-gel column chromatography (hexane/ethyl acetate = 2/1) to give **4** (136 mg, 47%) as the yellow solid.

HR-FD-MS (positive): $[\text{M}]^+$ Found m/z 283.10774, $\text{C}_{13}\text{H}_{17}\text{O}_6\text{N}$ requires m/z 283.10559; ^1H -NMR (270 MHz, CDCl_3 , rt): 11.54 (1H, s), 10.59 (1H, br s), 7.52 (1H, d, $J=8.9$ Hz), 7.08 (1H, br s), 6.51 (1H, d, $J=8.9$ Hz), 3.92 (3H, s), 1.54 (9H, s) ppm; ^{13}C -NMR (67.8 MHz, CDCl_3 , rt): 171.0, 156.3, 154.1, 153.5, 126.4, 113.9, 111.2, 104.4, 83.1, 52.1, 28.1 ppm; IR (neat): 3410, 2980, 1670, 1599, 1534, 1440, 1257, 1158, 1064 cm^{-1} ; mp: 180-200°C (decomposition).

Methyl 3-(*tert*-butoxycarbonylamino)-2-hydroxy-4-(methoxymethoxy)benzoate (5).

Compound **4** (231 mg, 0.815 mmol) was dissolved in DMF (10 mL) and diisopropylethylamine (0.421 mL, 2.44 mmol) was added. To this mixture, methoxymethyl chloride (0.067 mL, 0.896 mmol) was added and reacted for 2.5 hours under argon atmosphere. The reaction mixture was diluted with water and extracted by ethyl acetate. The organic layer was washed with brine, dried over sodium sulfate and evaporated. The residue was purified by silica-gel column chromatography (hexane/ethyl acetate = 3/1) to give **5** (198 mg, 74%) as the yellowish solid.

HR-FD-MS (positive): $[\text{M}]^+$ Found m/z 327.12895, $\text{C}_{15}\text{H}_{21}\text{O}_7\text{N}$ requires m/z 327.13180; ^1H -NMR (270 MHz, CDCl_3 , rt): 11.15 (1H, s, OH), 7.69 (1H, d, $J=8.9$ Hz), 6.70 (1H, d, $J=8.9$ Hz), 5.88 (1H, br s), 5.25 (2H, s), 3.92 (3H, s), 3.50 (3H, s), 1.50 (9H, s) ppm; ^{13}C -NMR (67.8 MHz, CDCl_3 , rt): 170.3, 158.1, 158.0, 153.7, 128.6, 114.7, 106.9, 105.8, 94.5, 79.8, 56.2, 51.9, 28.0 ppm; IR (neat): 3370, 2979, 1717, 1672, 1506, 1439, 1337, 1271, 1153, 1048 cm^{-1} ; mp: 142-144°C.

3-(*tert*-butoxycarbonylamino)-2-hydroxy-4-(methoxymethoxy)benzoic acid (6).

Compound **5** (207 mg, 0.632 mmol) was dissolved in methanol (6 mL) and water (2 mL) was added. To this mixture, aq. solution of 2 M sodium hydroxide (20 mL) was added and reacted for one day under argon atmosphere. The reaction mixture was acidified by 1 M hydrochloric acid, diluted with water and extracted by ethyl acetate. The organic layer was washed with brine, dried over sodium sulfate and evaporated to give **6** (119 mg, 60%) as the orange oil.

HR-FD-MS (positive): [M]⁺ Found *m/z* 313.11783, C₁₄H₁₉O₇N requires *m/z* 313.11615; ¹H-NMR (270 MHz, CDCl₃, rt): 7.80 (1H, d, *J*=8.9 Hz), 6.75 (1H, d, *J*=8.9 Hz), 6.50 (1H, br s), 5.27 (2H, s), 3.51 (3H, s), 1.52 (9H, s) ppm; ¹³C-NMR (67.8 MHz, CDCl₃, rt): 172.7, 158.20, 158.17, 157.96, 129.8, 114.2, 107.0, 105.6, 94.4, 81.0, 56.2, 27.9 ppm; IR (neat): 3293, 2980, 1672, 1506, 1267, 1136, 1050 cm⁻¹.

***N*-(2-(benzyloxycarbonylamino)ethyl)-**

3-(*tert*-butoxycarbonylamino)-2-hydroxy-4-(methoxymethoxy)benzamide (7a).

Compound **6** (223.0 mg, 0.712 mmol) was dissolved in DMF (12 mL) and diisopropylethylamine (0.184 mL, 1.424 mmol), EDCI (136.5 mg, 0.712 mmol), DMAP (cat.) were added. To this mixture, *N*-Cbz-ethylenediamine HCl (213.5 mg, 0.926 mmol) was added and reacted for one day under argon atmosphere. The reaction mixture diluted with water and extracted by ethyl acetate. The organic layer was washed with brine, dried over sodium sulfate and evaporated. The residue was purified by silica-gel column chromatography (chloroform/methanol = 20/1) to give **7** (170.0 mg, 49%) as the slightly yellow oil.

HR-FD-MS (positive): [M]⁺ Found *m/z* 489.21033, C₂₄H₃₁O₈N₃ requires *m/z* 489.21111; ¹H-NMR (270 MHz, CDCl₃, rt): 12.00 (1H, s, OH), 8.11 (1H, br s, NH), 7.62 (1H, d, *J*=8.9 Hz), 7.36-7.28 (5H, m), 6.70 (1H, d, *J*=8.9 Hz), 6.50 (1H, br s, NH), 5.45 (1H, br s, NH), 5.24 (2H, s), 5.11 (2H, s), 3.55 (2H, br t, *J*=6.0 Hz), 3.51 (3H, s), 3.44 (2H, br t, *J*=6.0 Hz), 1.53 (9H, s); ¹³C-NMR (67.8 MHz, CDCl₃, rt): 169.3, 157.3, 155.5, 155.0, 154.9, 136.5, 128.3, 127.9 (2C), 126.0, 115.0, 111.0, 105.3, 94.5, 80.9, 66.4, 56.2, 40.7, 40.1, 28.0 ppm; IR (neat): 3332, 2978, 1708, 1499, 1366, 1272, 1160, 1050 cm⁻¹.

3-amino-*N*-(2-benzyloxycarbonylaminoethyl)-2,4-dihydroxybenzamide (8a).

Compound **7** (127.9 mg, 0.262 mmol) was dissolved in 4 M hydrogenchloride/1,4-dioxane solution (5 mL) and reacted for 2 hours at 0°C under argon atmosphere. The reaction mixture was evaporated and the residue was purified by silica-gel column

chromatography (chloroform/methanol = 10/1) to give **8** (53.7 mg, 60%) as the slightly yellow oil.

HR-FD-MS (positive): [M]⁺ Found *m/z* 345.13056, C₁₇H₁₉N₃O₅ requires *m/z* 345.13247; ¹H-NMR (270 MHz, CD₃OD, rt): 7.63 (1H, d, *J*=8.9 Hz), 7.31-7.26 (5H, m), 6.52 (1H, d, *J*=8.9 Hz), 5.04 (2H, s), 3.47 (2H, t, *J*=5.8 Hz), 3.34 (2H, t, *J*=5.8 Hz); ¹³C-NMR (67.8 MHz, CD₃OD, rt): 171.8, 159.4, 158.0, 156.9, 138.4, 129.58, 129.52, 129.0, 128.8, 108.4, 107.5, 107.3, 67.4, 41.1, 40.6 ppm; IR (neat): 3291, 2941, 1654, 1603, 1557, 1282, 1100 cm⁻¹.

3-amino-*N*-(2-aminoethyl)-2,4-dihydroxybenzamide (2).

Compound **8** (53.7 mg, 0.157 mmol) was dissolved in methanol (3.5 mL) and Pd-C (25 mg) was added. The mixture was reacted for 30 min at 0°C under argon atmosphere. The reaction mixture was passed through the celite pad to give **2** (27.9 mg, 85%) as the slightly brown oil.

HR-FD-MS (positive): [M]⁺ Found *m/z* 211.09340, C₉H₁₃O₃N₃ requires *m/z* 211.09569; ¹H-NMR (270 MHz, CD₃OD, rt): 7.10 (1H, d, *J*=8.9 Hz), 6.35 (1H, d, *J*=8.9 Hz), 3.63 (2H, t, *J*=5.6 Hz), 3.14 (2H, t, *J*=5.6 Hz); ¹³C-NMR (67.8 MHz, CD₃OD, rt): 173.8, 152.6, 151.2, 123.5, 118.7, 107.7, 107.5, 41.2, 38.1 ppm; IR (neat): 3257, 3050, 1638, 1558, 1288, 1161, 1019 cm⁻¹.

***N*-(2-acetamidoethyl)-**

3-(*tert*-butoxycarbonylamino)-2-hydroxy-4-(methoxymethoxy)benzamide (7b).

Compound **6** (31.5 mg, 0.10 mmol) was dissolved in DMF (1.0 mL) and diisopropylethylamine (50 µL, 0.294 mmol), HOBt (15.1 mg, 0.123 mmol) and EDCI (31.6 mg, 0.165 mmol) was added. To this mixture, *N*-acetyleneethylenediamine (17.1 mg, 0.167 mmol) dissolved in DMF (0.5 mL) was added and stirred for 16 hrs at 40°C under argon atmosphere. The reaction mixture was diluted with 1 M hydrochloric acid and extracted by ethyl acetate. The organic layer was washed with sat. aq. sodium hydrogen carbonate, brine, dried over sodium sulfate and evaporated to give **7b** (19.1 mg, 48%) as the slightly yellow oil.

HR-FD-MS (positive): [M]⁺ Found *m/z* 397.18437, C₁₈H₂₇O₇N₃⁺ requires *m/z* 397.18490; ¹H-NMR (270 MHz, DMSO-*d*₆, rt): 13.25 (1H, s, OH), 8.80 (1H, t, *J*=5.1 Hz, NH), 8.00 (1H, t, *J*=5.1 Hz, NH), 7.85 (1H, s, NH), 7.68 (1H, d, *J*=9.1 Hz), 6.62 (1H, d, *J*=9.1 Hz), 5.21 (2H, s), 3.37 (3H, s), 3.36-3.28 (2H, m), 3.20 (2H, dt, *J* = 5.1, 5.8 Hz), 1.79 (3H, s), 1.39 (9H, s) ppm; ¹³C-NMR (67.5 MHz, DMSO-*d*₆, rt): 170.0, 169.8, 159.1, 157.2, 154.0, 125.8, 115.5, 108.8, 104.8, 93.9, 78.0, 55.7, 38.9, 38.0, 28.0, 22.5 ppm; IR (neat): 3320,

2975, 2930, 1700, 1641, 1595, 1498, 1457, 1366, 1274, 1159, 1048 cm⁻¹.

***N*-(2-acetamidoethyl)-3-amino-2,4-dihydroxybenzamide (8b).**

Compound **7b** (6.2 mg, 15.6 μmol) was dissolved in 1.25 M HCl/methanol (0.5 mL) and stirred for 7 hrs. The reaction mixture was dried and purified by preparative thin layer chromatography (chloroform/methanol = 5/1, 2% acetic acid) to give **8b** (3.8 mg, 78%) as the slightly brown oil of acetic acid salt.

HR-ESI-MS (positive): [M+H]⁺ Found *m/z* 254.1125, C₁₁H₁₆O₄N⁺ requires *m/z* 254.1141; ¹H-NMR (500 MHz, CD₃OD, rt): 7.00 (1H, d, *J*=8.7 Hz), 6.32 (1H, d, *J*=8.7 Hz), 3.44 (2H, t, *J*=5.9 Hz), 3.36 (2H, t, *J*=5.9 Hz), 1.93 (3H, s) ppm; ¹³C-NMR (67.5 MHz, CD₃OD, rt): 174.2, 173.0, 152.5, 150.8, 123.7, 118.1, 108.1, 107.3, 40.3, 40.1, 22.5 ppm; IR (neat): 3327, 3099, 2939, 1635, 1558, 1545, 1497, 1459, 1435, 1363, 1285 cm⁻¹.

Affinity gel 9.

NHS-activated Sepharose™ (2 mL, GE Healthcare Co.) was filled in a plastic column and washed with 1 mM hydrochloric acid (25 mL) and then replaced with ligand **2** (28 mg) dissolved in 1 mL of coupling buffer (0.2 M NaHCO₃, 0.5 M NaCl, pH 8.3). After reacting for 2 hours at room temperature, the column was washed with 6 mL of coupling buffer and subsequently with 12 mL of blocking buffer (0.5 M ethanolamine, 0.5 M NaCl, pH 8.3). After an hour of blocking reaction, the column was washed with 0.1 M Tris-HCl buffer (pH 8.5) and 0.1 M acetate buffer with 0.5 M NaCl (pH 4.0) and then used. The residual **2** and the released NHS in the coupling buffer was quantified by HPLC to determine the amount of ligand on the gel.

HPLC conditions: Column- Inertsustain C18, φ4.6×250 mm (GL Science Inc.); eluent- 50% aq. Methanol, 0.1% formic acid; flow rate- 0.8 mL/min; detection wave length- 254 nm (for detection of remaining ligand **2**); Column- Inertsustain C18, φ4.6×250 mm (GL Science Inc.); eluent- 5% Methanol in 10 mM ammonium acetate buffer (pH5.7); flow rate- 0.8 mL/min; detection wave length- 254 nm (for detection of liberated NHS)

4.3. Preparation of crude enzyme from rat intestinal acetone powder.

Rat intestinal acetone powder (5 g) was homogenized in 100 mL of 0.1 M phosphate buffer (pH 7.0, 5 mM EDTA), centrifuged (15,000 g × 30 min) and the supernatant was collected. For maltase assay, the supernatant was diluted four times by 0.1 M phosphate buffer (pH 6.3) and used as the crude enzyme. For sucrase assay, the supernatant was directly used as the crude enzyme. Sucrase activity and maltase activity was designated as an activity of sucrose and maltose hydrolyzing activity in the

crude enzyme.

4.4. Intestinal α -glucosidase activity determination.

Substrate (300 μ L; 56 mM sucrose or 3.5 mM maltose in 0.1 M phosphate buffer, pH 6.3) was pre-incubated at 37°C for 5 min. Crude enzyme (200 μ L) was added and reacted for 20 min at 37°C. The reaction was stopped by adding 0.75 mL of 2 M Tris-HCl buffer (pH 7.0) and the amount of liberated glucose was measured by glucose oxidase method using Glucose CII-test Wako (Wako Pure Chem. Co.). One unit was defined as an enzyme that hydrolyzed 1 μ mol of substrate per minute. Experiments are done in duplicate and repeated at least three times and the representative result is shown in the figures.

4.5. Intestinal α -glucosidase inhibitory activity determination.

Sample solution dissolved in DMSO (50 μ L) and substrate (250 μ L; 56 mM sucrose or 3.5 mM maltose in 0.1 M phosphate buffer, pH 6.3) was pre-incubated at 37°C for 5 min. Crude enzyme (200 μ L) was added and reacted for 20 min at 37°C. The reaction was stopped by adding 0.75 mL of 2 M Tris-HCl buffer (pH 7.0) and the amount of liberated glucose was measured by glucose oxidase method using Glucose CII-test Wako (Wako Pure Chem. Co.). Experiments are done in duplicate and repeated at least three times and the representative result is shown in the figures. The K'_i was calculated from the non-linear regression of Michaelis-Menten equation which was analyzed by Prism 6 (GraphPad Software, Inc.).

4.6. Affinity purification of maltase by gel 9.

Crude enzyme (1 mL) diluted with 2 mL of 3.5 mM maltose (0.1 M phosphate buffer, 5 mM EDTA, pH7.0) was added to the column filled with gel 9 (2 mL). The column was washed with 30 mL of washing buffer (3.5 mM maltose, 0.1 M phosphate buffer, 5 mM EDTA, pH7.0) and then eluted by elution buffer A (0.1 M phosphate buffer, 5 mM EDTA, pH7.0) and elution buffer B (1 M NaCl, 0.1 M phosphate buffer, 5 mM EDTA, pH7.0). Each elution was fractionated, desalted by HiTrap Desalting column (GE Healthcare Co.), concentrated by Amicon Ultra-0.5 Centrifugal Filter Devices (Millipore). Protein concentration of the fractions was quantified by Quick start protein assay (Bio-rad Co.) and the glucosidase activity was measured. For the experiment of Table 1, elution buffer A was skipped.

4.7. Analysis of purified fraction

Samples were mixed with 3 $\times$ Laemmli buffer and heated at 95°C for 5 min. SDS-PAGE was performed with 7.5% gel and visualized by silver staining. Native-PAGE was performed following the procedure of SDS-PAGE without SDS and denaturing step. The obtained band of Native-PAGE

gel was cut, extracted and the glucosidase activity was measured. Western blotting was performed by transferring to PVDF membrane (Hybond-P, GE Healthcare Co.). Following antibodies were used: Primary antibody, Maltase-glucoamylase (H-58) (Santa Cruz biotechnology Co.) dilution 1/1000, secondary antibody, anti-rabbit IgG-HRP (Santa Cruz biotechnology Co.) dilution 1/20000. ECL Prime Western blotting detection reagents (GE Healthcare Co.) was used to visualize the protein and was captured with LumiVision PRO 400EX (Aisin seiki Co., Ltd.).

Acknowledgments

The authors thank Mr. Yusuke Takata and Dr. Eri Fukushi of the GC-MS and NMR Laboratory, Faculty of Agriculture, Hokkaido University, for their skillful measurements of mass spectra.

Supporting material available: Michaelis-Menten equation of **8b** and NMR spectra of synthetic compounds.

REFERENCES

1. Hage, D. S.; Anguizola, J. A.; Bi, C.; Li, R.; Matsuda, R.; Papastavros, E.; Pfaunmiller, E.; Vargas, J.; Zheng, X. *J. Pharm. Biomed. Anal.* **2012**, *69*, 93-105.
2. Urh, M.; Simpson, D.; Zhao, K. *Methods Enzymol.* **2009**, *463*, 417-38.
3. Terpe, K. *Appl. Microbiol. Biotechnol.* **2003**, *60*, 523-33.
4. Zhao, X.; Li, G.; Liang, S. *J. Anal. Methods Chem.* **2013**, *2013*, 581093.
5. Block, H.; Maertens, B.; Spriestersbach, A.; Brinker, N.; Kubicek, J.; Fabis, R.; Labahn, J.; Schäfer, F. *Methods Enzymol.* **2009**, *463*, 439-473.
6. Cheung, R. C.; Wong, J. H.; Ng, T. B. *Appl. Microbiol. Biotechnol.* **2012**, *96*, 1411-1420.
7. Guan, D.; Chen, Z. *Biotechnol. Lett.* **2014**, *36*, 1391-406.
8. Kato, E.; Sasaki, T.; Ueda, M. *Bioorg. Med. Chem.* **2008**, *16*, 4600-16.

9. Lu, M.; Ngo, W.; Mei, Y.; Munshi, V.; Burlein, C.; Loughran, M. H.; Williams, P. D.; Hazuda, D. J.; Miller, M. D.; Grobler, J. A.; Diamond, T. L.; Lai, M. T. *Protein Expr. Purif.* **2010**, *71*, 231-9.
10. Ogata, M.; Kameshima, Y.; Hattori, T.; Michishita, K.; Suzuki, T.; Kawagishi, H.; Totani, K.; Hiratake, J.; Usui, T. *Carbohydr. Res.* **2010**, *345*, 2623-9.
11. Valsasina, B.; Kalisz, H. M.; Isacchi, A. *Expert Rev. Proteomics* **2004**, *1*, 303-15.
12. Wan, Y.; Hur, W.; Cho, C. Y.; Liu, Y.; Adrian, F. J.; Lozach, O.; Bach, S.; Mayer, T.; Fabbro, D.; Meijer, L.; Gray, N. S. *Chem. Biol.* **2004**, *11*, 247-59.
13. Gao, H.; Kawabata, J. *Bioorg. Med. Chem. Lett.* **2008**, *18*, 812-5.
14. Arsiningtyas, I. S.; Gunawan-Puteri, M. D.; Kato, E.; Kawabata, J. *Nat. Prod. Res.* **2014**, *28*, 1350-3.
15. Krentz, A. J.; Bailey, C. J. *Drugs* **2005**, *65*, 385-411.
16. Yoshikawa, M.; Murakami, T.; Shimada, H.; Matsuda, H.; Yamahara, J.; Tanabe, G.; Muraoka, O. *Tetrahedron Lett.* **1997**, *38*, 8367-8370.
17. Lee, L. M.; Salvatore, A. K.; Flanagan, P. R.; Forstner, G. G. *Biochem. J.* **1980**, *187*, 437-46.
18. Flanagan, P. R.; Forstner, G. G. *Biochem. J.* **1978**, *173*, 553-563.
19. Kato, E.; Oikawa, K.; Takahashi, K.; Kawabata, J. *Biosci. Biotechnol. Biochem.* **2012**, *76*, 1044-6.
20. Dahlqvist, A.; Telenius, U. *Biochem. J.* **1969**, *111*, 139-46.
21. Quezada-Calvillo, R.; Rodriguez-Zuniga, F.; Underdown, B. J. *Biochem. Biophys. Res. Commun.* **2002**, *295*, 394-400.

Supplementary material

Selective purification of intestinal maltase complex by affinity chromatography employing an uncompetitive inhibitor as the ligand

Eisuke Kato,* Hiroki Tsuji and Jun Kawabata*

Laboratory of Food Biochemistry, Division of Applied Bioscience, Graduate School of Agriculture,
Hokkaido University, Kita-ku, Sapporo, Hokkaido 060-8589, Japan

*Corresponding author:

E. Kato eikato@chem.agr.hokudai.ac.jp; J. Kawabata junk@chem.agr.hokudai.ac.jp

Supplementary Figure 1. Michaelis-Menten equation of **8b**.

This report was created by ACD/NMR Pro Frequency Academic Edition. For more information go to www.acdatools.com/nmrpro/							
Acquisition Time (sec)	0.0351	Date	20 Oct 2011 17:00:08	Date Stamp	Thu Oct 20 16:59:19 2011	Name of Transients	16
	D:\90MHz\NMR4\Hals					Solvent	CHLOROFORM-d
Original Points Count	16384	Points Count	16384	Pulse Sequence	NON	Receiver Gain	15.00
Spectrum Offset (Hz)	498.0697	Sweep Width (Hz)	504.76	Temperature (degrees C)	25.000

<p>Report was created by ACORN/INRA Academic Edition. For more information go to http://www.acornplus.com/infor/</p>							
Acquisition Time (sec)		11 Oct 2014		11:39:22		Date Stamp	Sat Oct 11 11:39:11 2014
File Name	D:\NMR2\8-36-2CALS	Frequency (MHz)	67.80	NUC1	13C	Number of Transients	256
Original Points Count	32768	Pulse Sequence	BCM	Receiver Gain	26.00	Solvent	CHLOROFORM-d
Spectrum Offset (Hz)	6819.2954	Spins Width (Hz)	18306.64	Temperature (degree C)	26.500

19

This report was created by ACD/NNIR Processor Academic Edition. For more information go to www.accurials.com/nnirproc/

Acquisition Time (sec)	Time	Date	28 Jul 2014 18:48:40	Date Stamp	18/Oct/2011 14:55:13
File Name	D:\400nm\11\11-11-11\NNMR5_Hals			Nucleus	
Original Points Count	16394	Pulses Count	16394	Receiver Gain	18.00
Spectra Offset (Hz)	1639.3994	Scan Width (Hz)	1401.76	Number of Transients	16
		Temperature (degree C)	26.00	Solvent	CHLOROFORM-d

Acquisition Time (sec)	1.7890	Date	28 Jul 2014 18:48:58	Date Stamp	18/01/2011 15:10:59
2D NMR Experiment	DQNP130YD	Sample Name	13C-15NMRVal5_Cal	Frequency (MHz)	67.80
Original Points Count	32768	Points Count	32768	Pulse Sequence	BCM
Spectrum Offset (Hz)	68103580	Spectra Width (Hz)	18306.64	Temperature (degree C)	26.500
				Receiver Gain	27.00
				Solvent	CHLOROFORM-d
				Number of Transients	102

20

Supplementary Figure 4. ¹H and ¹³C NMR of compound **6**.

This report was created by ACD/NMR Processor (Academic Edition). For more information go to www.acutials.com/nmrproc

Acquisition Time (sec)	3:03:31	Date	28 Jul 2014 16:01:59	Date Stamp	22/Nov/2013 19:09:39
File Name	D:\90Mhz\90Mhz\NMR7_Hals	Frequency (MHz)	270.05	Nucleus	¹³ C
Original Points Count	16384	Points Count	16384	Pulse Sequence	NON
Spectrum Offset (Hz)	4919.3885	Sweep Width (Hz)	5401.76	Temperature (degrees C)	24.900
				Receiver Gain	21.00
				Solvent	CHLOROFORM-d
				Number of Transients	16

7a_Hesp

VerticalScaleFactor = 1

Normalized Intensity

Chemical Shift (ppm)

No.	(ppm)	Value
1	[0.02..0.02]	1.00
2	[1.48..1.57]	10.57
3	[3.38..3.61]	8.47
4	[5.07..5.18]	2.48
5	[5.21..5.32]	2.51
6	[5.40..5.49]	0.82
7	[6.46..6.53]	0.75
8	[6.67..6.74]	1.18
9	[7.27..7.44]	5.66
10	[7.58..7.66]	1.00
11	[8.07..8.16]	0.97
12	[11.97..12.03]	0.77

No.	(ppm)	(Hz)	Height
1	0.00	0.0	0.1259
2	1.26	338.9	0.0894
3	1.53	413.1	1.0000
4	1.65	444.8	0.1579
5	2.88	777.8	0.2123
6	2.95	797.3	0.2271
7	3.44	929.1	0.0461
8	3.51	946.9	0.4130
9	3.53	954.2	0.0603
10	3.54	956.5	0.0544
11	3.56	962.4	0.0430
12	5.11	1379.2	0.1584

No.	(ppm)	(Hz)	Height
13	5.24	1415.8	0.2302
14	5.45	1470.5	0.0175
15	6.50	1754.1	0.0246
16	6.68	1803.6	0.0584
17	6.71	1812.1	0.0634
18	7.27	1962.1	0.1464
19	7.30	1972.0	0.0491
20	7.31	1975.0	0.0456
21	7.33	1980.0	0.1834
22	7.35	1984.6	0.0851
23	7.60	2051.5	0.0276
24	7.63	2060.4	0.0275

No.	(ppm)	(Hz)	Height
25	8.11	2190.6	0.0205
26	12.00	3240.1	0.0259

Acquisition Time (sec)	1.7890	Date	28 Jul 2014 16:03:20	Date Stamp	28 Jul 2014 13:15:38
File Name	D:\0000\000000\000000\NMR\NMR\Wt_01	Frequency (MHz)	67.80	Number of Transients	200
Original Points Count	32768	Points Count	32768	Pulse Sequence	BCM
Spectrum Offset (Hz)	6808.6802	Sweep Width (Hz)	18306.64	Temperature (degree C)	25.800
				Receiver Gain	26.00
				Solvent	CHLOROFORM-d

7_C.als VerticalScaleFactor = 1

No.	(ppm)	(Hz)	Height
1	27.95	1895.3	1.0000
2	40.07	2716.5	0.1212
3	40.64	2755.7	0.1324
4	56.16	3807.7	0.3361
5	66.44	4504.4	0.2041

No.	(ppm)	(Hz)	Height
6	76.53	5188.8	0.5930
7	77.00	5220.6	0.5994
8	77.21	5234.6	0.2340
9	77.48	5253.0	0.5841
10	80.85	5481.5	0.1320

No.	(ppm)	(Hz)	Height
11	94.54	6410.1	0.1828
12	105.34	7141.9	0.2031
13	110.99	7525.2	0.0695
14	114.95	7793.9	0.1345
15	126.00	8542.6	0.1228

No.	(ppm)	(Hz)	Height
16	127.68	8670.0	0.7342
17	128.34	8701.2	0.6925
18	136.53	9256.6	0.1630
19	154.87	10500.2	0.0852
20	154.97	10506.9	0.1175

No.	(ppm)	(Hz)	Height
21	155.46	10540.5	0.0712
22	157.25	10661.7	0.1041
23	169.34	11481.3	0.0966

Supplementary Figure 6. ¹H and ¹³C NMR of compound **8a**.

Supplementary Figure 7. ¹H and ¹³C NMR of compound 2.

Supplementary Figure 8. ¹H and ¹³C NMR of compound 7b.

Supplementary Figure 9. ^1H and ^{13}C NMR NMR of compound **8b**.