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Orthogonality of α-Sulfoquinovosidase in Human Cells and Development of Its Fluorescent Substrate

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Human orthogonal enzymes (HOEs) do not show the same activities as the endogenous enzymes of human cells and thus are useful as amplification enzymes to detect antigen proteins in biological samples. Here, we evaluate a new HOE from *Escherichia coli*, α-sulfoquinovosidase (α-SQase). We confirmed that the activity of α-SQase did not exist in examined human cell lines, and thus it was applicable to live-cell enzyme-linked immunosorbent assay (ELISA) in which the antigen membrane protein on cells was detected without inactivating endogenous enzymes, a pretreatment required for cell ELISA using conventional amplification enzymes. Here, we also developed a fluorescent substrate for α-SQase whose active residue is located at the end of the narrow, deep pocket of the substrate recognition site. The designed methylumbelliferyl substrate with a hydroxyl benzyl alcohol linker showed a similar reactivity to the *p*-nitrophenol substrate, a good substrate for α-SQase.

1. Introduction

Amplification enzymes are used for the detection of antigens in bioanalytical methods, such as Western blotting, enzyme-linked immunosorbent assay (ELISA), and immunohistochemistry. These enzymes are conjugated with the antibodies of the target antigens. Horseradish peroxidase,[(1,2)] alkaline phosphatase,[(3)] and β-galactosidase[(4)] are conventionally used enzymes.

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Because these conventional enzymes are endogenous substances of human cells,[5–8] pretreatment is required to inactivate the endogenous enzymes. This treatment not only induces cell death but also denatures the target antigen, which may impede the recognition of antigens by antibodies.

To address this issue, we proposed a new class of amplification enzymes, named human cell orthogonal enzymes (HOEs), whose activities do not exist in human cells. We previously showed that α-arabinofuranosidase[9] and β-galacturonidase[10] are effective HOEs for the detection of antigens in live human cells.

Here, we examined α-sulfoquinovosidase (α-SQase) as a new HOE. α-SQase is a glycohydrolase found in bacteria. Natural substrates of α-SQase are diacylglyceride or glycerol derivatives of sulfoquinovose.[11,12] α-SQase from *Escherichia coli* has relatively high reactivity toward the *p*-nitrophenol substrate at neutral pH; specifically, its reactivity is comparable to those of conventional enzymes.[12–15] However, chromogenic substrates have limited utility in crude cell lysates where high background absorption provides poor sensitivity. Furthermore, α-SQase was found to show poor reactivity toward the methylumbelliferyl (MU) substrate probably because the large methylumbelliferyl substrate is sterically hindered from binding to the active residue in the pocket of the substrate recognition site of α-SQase.[16] Therefore, another purpose of the study was to develop a fluorescent substrate that can react with α-SQase.

2. Data, Materials, and Methods

2.1 Materials and general procedure

All chemical reagents and solvents for synthesis were purchased from commercial suppliers (Wako Pure Chemical, Tokyo Kasei Industry, and Sigma-Aldrich Japan, Japan) and were used without further purification. All solvents for high-performance liquid chromatography (HPLC) were purchased from Wako Pure Chemical. The composition of mixed solvents is given here as volume ratios (v/v). ^{1}H NMR spectra were recorded on an AVANCE III HD (400 MHz for ^{1}H, Bruker, Massachusetts, United States of America) at room temperature. Column chromatography purification was performed on an EPCLC-W-Prep 2XY chromatograph (Yamazen, Osaka, Japan). HPLC purification was performed on a Hitachi Chromaster using a YMC-Pack PolymerC18 (150 mm × 4.6 mm × 4 μm, YMC, Kyoto, Japan).

2.2 Synthesis of 2

Fluorescent substrate 1 was synthesized following Fig. 1. Penta-O-acetyl-β-D-glucopyranose (3.90 g, 10 mmol) was dissolved in dehydrated DCM (20 mL) under nitrogen atmosphere, acetic acid (1.049 mL, 0.0346 mol) was added, and the mixture was cooled to 0 ℃ in an ice bath. Thionyl chloride (5 mL, 0.0688 mol) was then added dropwise to the reaction mixture in an ice bath and stirred at room temperature for 18 h. The mixture was concentrated under reduced pressure at 30 ℃ and then azeotroped with toluene (2 × 10 mL) to obtain compound 2 as a white solid.

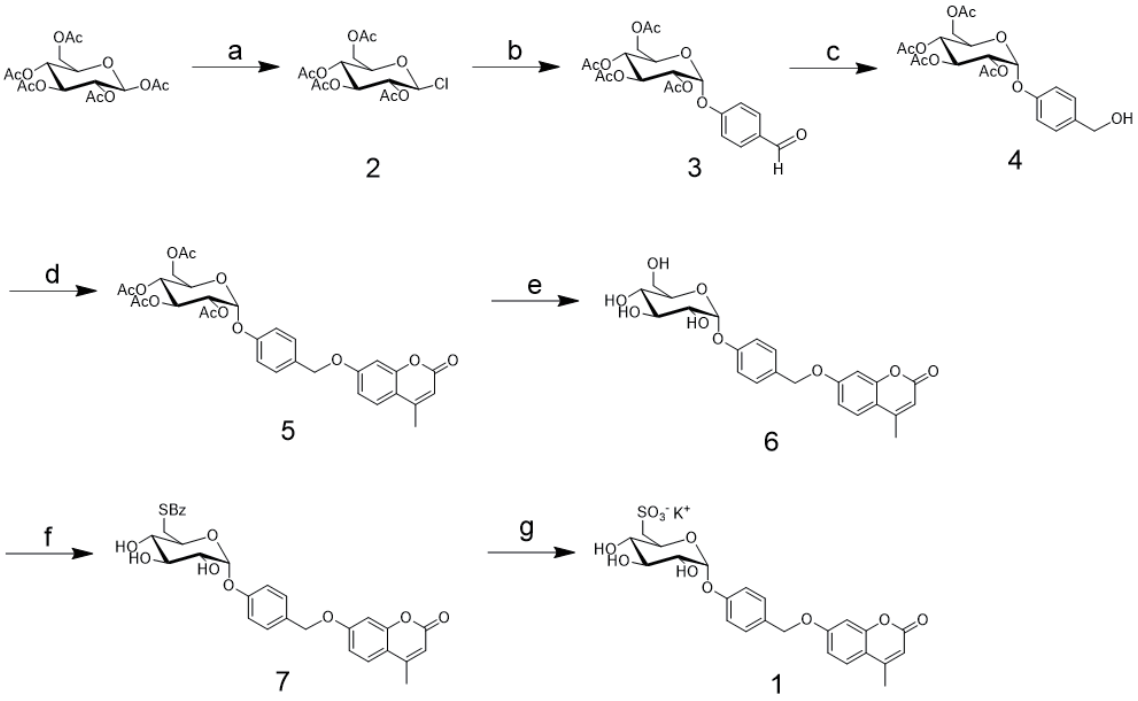

Fig. 1. Synthesis of linker-type fluorescent substrate. (a) $SOCl_2$, AcOH, CH_2Cl_2; (b) tetrabutylammonium, 4-formylphenolate, MeCN; (c) $NaBH_4$, MeOH; (d) methylumbelliferyl, tributylphosphine, 1,1-(azodicarbonyl) dipiperidine; (e) CH_3ONa, MeOH; (f) thiobenzoic acid, triphenylphosphine, diisopropyl azodicarboxylate, tetrahydrofuran; (g) H_2O_2, AcOH, KOAc.

^{1}H NMR (400 MHz, $CDCl_3$) δ 2.00–2.14 (12 H, m, Ac), δ 3.82 (1 H, ddd, J5,6a = 2.2, J5,6b = 4.8, J4,5 = 10.0 Hz, H5), δ 4.18 (1 H, dd, J6a,6b = 6.0 Hz, H6a), δ 4.28 (1 H, dd, H6b), δ 5.15–5.22 (3 H, m, H2,3,4), δ 5.31 (1 H, d, J1,2 = 8.0 Hz, H1).

2.3 Synthesis of 3

4-Hydroxybenzaldehyde (1.22 g, 10.0 mmol) and tetrabutylammonium hydroxide (40% in water, 6.389 mL, 9.8 mmol) were dissolved in methanol (5 mL) under nitrogen atmosphere and stirred at room temperature for 1 h. The mixture was concentrated under reduced pressure, azeotroped with toluene (2 × 10 mL), and lyophilized to obtain tetrabutylammonium 4-formylphenolate (2.971 g, 8.18 mmol) as a yellow-white solid. Compound 2 was dissolved in dehydrated acetonitrile (30 mL) under nitrogen atmosphere and added dropwise to a solution of tetrabutylammonium 4-formylphenolate (2.971 g, 8.18 mmol) in dehydrated acetonitrile (470 mL). The mixture was stirred for 3 days, concentrated under reduced pressure at 30 °C, and dissolved in DCM (100 mL). The solution was washed three times with 5% NaOH solution (100 mL), washed with brine, and dried over Na_2SO_4. After filter filtration through Na_2SO_4, the mixture was concentrated and purified by column chromatography (silica gel 60, 0.063–0.200 nm) (ethyl acetate/hexane = 2/3) to obtain compound 3 (0.30 g, 0.66 mmol) as a pale yellow viscous liquid.

^{1}H NMR (400 MHz, $CDCl_3$) δ 2.00–2.14 (12 H, m, Ac), δ 4.02–4.33 (3 H, m, H5,6), δ 5.06–5.22 (2 H, m, H2,4), δ 5.71 (1 H, m, H3), δ 4.34 (1 H, d, J1,2 = 4.0 Hz, H1), δ 7.24 (2 H, m, H2,6-Ph), δ 7.88 (2 H, m, H3,5-Ph), δ 9.88 (1 H, s, Ph-CHO).

2.4 Synthesis of 4

Compound 3 (0.30 g, 0.66 mmol) was dissolved in methanol (20 mL) and cooled to 0 ℃ in an ice bath. Sodium borohydride (15 mg, 0.40 mmol) was added to the solution and stirred at 0 ℃ for 2 h. 1 M HCl was added to quench the reaction. The mixture was extracted with ethyl acetate. The organic phase was washed with saturated sodium bicarbonate solution and brine and dried over Na_2SO_4. After filter filtration through Na_2SO_4, the mixture was concentrated and purified by column chromatography (silica gel 60, 0.063–0. 200 nm) (ethyl acetate/hexane = 1/1) to obtain compound 4 (0.20 g, 0.44 mmol) as a pale yellow viscous liquid.

^{1}H NMR (400 MHz, $CDCl_3$) δ 2.00–2.10 (13 H, m, Ac), δ 4.04 (1 H, dd, J6a,6b = 12.4, J5,6a = 2.4 Hz, H6a), δ 4.12 (1 H, ddd, J5,6b = 4.4, J4,5 = 10.0 Hz, H5), δ 4.25 (1 H, dd, H6b), δ 4.63 (2 H, s, CH2), δ 5.04 (1 H, dd, J1,2 = 3.6, J2,3 = 10.8 Hz, H2), δ 5.16 (1 H, dd, J3,4 = 10.0 Hz, H4), δ 5.70 (1 H, dd, H3), δ 5.73 (1 H, d, H1), δ 7.07 (2 H, m, H2,6-Ph), δ 7.31 (2 H, m, H3,5-Ph).

2.5 Synthesis of 5

Triphenylphosphine (116 mg, 0.44 mmol) was dissolved in dehydrated THF (2 mL) under nitrogen atmosphere and cooled to 0 ℃ in an ice bath (Solution C). Diisopropyl azodicarboxylate (86 μL, 0.44 mmol) was dissolved in dehydrated THF (1 mL) under nitrogen atmosphere, cooled to 0 ℃ in an ice bath, and added dropwise to solution C without exceeding 5 ℃. The mixture was then stirred at 0 ℃ for 30 min to obtain triphenylphosphine and diisopropyl azodicarboxylate as a yellow-white mixture (Solution D). Compound 4 (0.20 g, 0.44 mmol) and 4-methylumbelliferone (77.5 mg, 0.44 mmol) were dehydrated in THF (2.0 mL) under nitrogen atmosphere. The mixture was added dropwise to solution D without exceeding 5 ℃. The mixture was slowly warmed to room temperature and stirred overnight. The mixture was concentrated and purified by column chromatography (silica gel 60, 0.063–0.200 nm) (ethyl acetate/hexane = 1/1) to obtain compound 5 (100 mg, 0.22 mmol) as a colorless viscous liquid.

^{1}H NMR (400 MHz, $CDCl_3$) δ 2.00–2.10 (13 H, m, Ac), δ 2.40 (3 H, s, Me-Ar), δ 4.05 (1 H, dd, J6a,6b = 12.4, J5,6a = 2.4 Hz, H6a), δ 4.12 (1 H, ddd, J5,6b = 4.4, J4,5 = 10.0 Hz, H5), δ 4.27 (1 H, dd, H6b), δ 5.05 (1 H, dd, J1,2 = 3.6, J2,3 = 10.8 Hz, H2), δ 5.08 (2 H, s, Ar-CH2-), δ 5.16 (1 H, dd, J3,4 = 10.0 Hz, H4), δ 5.71 (1 H, dd, H3), δ 5.76 (1 H, d, H1), δ 6.14 (1 H, s, H3-Ar), δ 6.87 (1 H, d, J6,8 = 2.0 Hz, H8-Ar), δ 6.92 (1 H, dd, J6,5 = 8.6 Hz, H6-Ar), δ 7.13 (2 H, m, H2,6-Ph), δ 7.39 (2 H, m, H3,5-Ph), δ 7.51 (1 H, d, H5-Ar).

2.6 Synthesis of 6

Compound 5 (100 mg, 0.22 mmol) was dissolved in dehydrated methanol (10 mL) under dehydrated atmosphere and cooled to 0 ℃ in an ice bath. 5.0 M sodium methoxide (1 drop) was added dropwise to the mixture and stirred at 0 ℃ for 3 h without exceeding pH 10. The mixture was then neutralized with acetic acid (10% in water), concentrated, and purified by column chromatography (silica gel 60, 0.063–0.200 nm) (ethyl acetate/methanol = 100/0 to 50/50) to obtain compound 6 (80 mg, 0.18 mmol) as a colorless viscous liquid.

^{1}H NMR (400 MHz, CD_3OD) δ 2.41 (3 H, s, Me-Ar), δ 3.43 (1 H, dd, J5,4 = J3,4 = 9.1 Hz, H4), δ 3.58 (1 H, dd, J1,2 = 3.7, J2,3 = 9.6 Hz, H2), δ 3.62–3.79 (3 H, m, H5 H6a H6b), δ 3.87 (1 H, dd, J3,4 = J2,3 = 9.1 Hz, H3), δ 5.09 (2 H, s, Ar-CH2-), δ 5.50 (1 H, d, J1,2 = 3.7Hz, H1), δ 6.13 (1 H, s, H3-Ar), δ 6.92 (1 H, d, J6,8 = 2.3 Hz, H8-Ar), δ 6.98 (1 H, dd, J6,5 = 8.7 Hz, H6-Ar), δ 7.20 (2 H, m, H2,6-Ph), δ 7.39 (2 H, m, H3,5-Ph), δ 7.64 (1 H, d, H5-Ar).

2.7 Synthesis of 7

Triphenylphosphine (92 mg, 0.35 mmol) was dissolved in dehydrated THF (1.6 mL) under nitrogen atmosphere and cooled to 0 ℃ in an ice bath (solution E). Diisopropyl azodicarboxylate (70 μL, 0.36 mmol) was dissolved in dehydrated THF (0.8 mL) under nitrogen atmosphere, cooled to 0 ℃ in an ice bath, and added dropwise to solution E without exceeding 5 ℃. The mixture was then stirred at 0 ℃ for 30 min to obtain triphenylphosphine and diisopropyl azodicarboxylate as a yellow-white mixture (solution F). Compound 6 (80 mg, 0.18 mmol) and thiobenzoic acid (77.5 mg, 0.44 mmol) were dissolved in dehydrated THF (1.6 mL) under nitrogen atmosphere and added dropwise to solution F without exceeding 5 ℃. The mixture was slowly warmed to room temperature and stirred overnight. The mixture was then concentrated and purified by column chromatography (silica gel 60, 0.063–0.200 nm) (ethyl acetate/hexane = 1/1) to obtain compound 7 as a colorless viscous liquid.

2.8 Synthesis of 1

Compound 7 (0.1 g), AcOH (3.5 mL), AcOK (82 mg, 0.84 mol), and 30% H_2O_2 (0.7 mL) were stirred at 50 ℃ for 24 h. The mixture was then diluted 5 times with pure water, and a diethyl ether solution of PPh_3 (1.0 M, 6 mL) was added to quench the reaction. The aqueous and organic phases of the reaction mixture were each collected. The organic phase was extracted twice with pure water. The aqueous phase used for extraction and the aqueous phase of the reaction solution were concentrated and purified by column chromatography (silica gel 60, 0.063–0.200 nm) (EtOAc/MeOH/H_2O = 7/2/1) and HPLC (H_2O/CH_3CN = 85/15 to 60/40) to obtain compound 1 as a white solid.

^{1}H NMR (400 MHz, D_2O) δ 2.42 (3 H, s, Me-Ar), δ 3.22 (H4), δ 3.50 (1 H, dd, J6a,6b = 14.9, J5,6a = 8.5 Hz, H6a), δ 4.05 (1 H, dd, J5,6b = 3.0 Hz, H6b), δ 4.36 (1 H, ddd, H5), δ 4.32 (1 H, dd,

J2,3 = 10.5, J1,2 = 3.7 Hz, H2), δ 4.39 (1 H, dd, J2,3 = 10.5, J3,4 = 5.0 Hz, H2), δ 5.16 (2 H, s, Ar-CH2-), δ 5.63 (1 H, d, H1), δ 6.13 (1 H, s, H3-Ar), δ 7.01-7.06 (2 H, m, H8-Ar H6-Ar), δ 7.20 (2 H, m, H2,6-Ph), δ 7.39 (2 H, m, H3, 5-Ph), δ 7.64 (1 H, d, H5-Ar).

2.9 Cell culture

JY25 (lymphoblastoid human B cell, provided by Dr. Taro Kinoshita) was grown in RPMI 1640 medium. HeLa (human cervical cancer cell lines, RIKEN BCR, Ibaraki, Japan), A549 (human alveolar adenocarcinoma cell lines, RIKEN BCR), and HepG2 (human liver cancer cell lines, JCRB Cell Bank, Osaka, Japan) were grown in Dulbecco's modified Eagle's medium (DMEM). Both media contained 10% fetal bovine serum (FBS). All cultures were maintained in a humidified atmosphere of 5% CO_2 and 95% air at 37 ℃.

2.10 Evaluation of endogenous activity of enzymes in cell lines

Potassium 4-nitrophenyl 6-deoxy-6-sulfonato-α-D-glucopyranoside (NPSQ) was synthesized according to a reported method.[12] For cell surface analysis, JY25 and HeLa cells suspended in 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffer (pH 7.4) were added to 96-well plates. Then, 3.0 mM *p*-nitrophenol substrates of alkaline phosphatase (AP), β-galactosidase (β-gal), and α-SQ in 10 mM HEPES (pH 7.4) were added and reacted at 37 ℃ for 18 h. The absorbance at 410 nm was then measured using a plate reader (Infinite 200PRO M Plex, TECAN, Männedorf, Switzerland), and the reaction ratios of the substrates were calculated. For intracellular analysis, JY25 and HeLa cells were lysed by adding RIPA buffer with stirring and then incubated for 15 min in an ice bath. The cell lysates were diluted nine times with 10 mM HEPES buffer (pH 7.4) and 0.1 M citrate buffer (pH 4.5). Each diluted lysate was added to 96-well plates, and 3.0 mM *p*-nitrophenol substrates of AP, β-gal, and α-SQ in 10 mM HEPES buffer (pH 7.4) were added and reacted at 37 ℃ for 18 h. The absorbance at 410 nm was then measured using a plate reader (Infinite 200PRO M Plex, TECAN), and the reaction ratios of the substrates were calculated.

2.11 Enzyme expression

The expression vector for α-SQase was constructed by inserting a synthetic DNA strand (synthesized by and purchased from Integrated DNA Technologies) coding α-SQase into the cloning site of the pET22b+ plasmid using In-Fusion HD Cloning Kit (Takara Bio Inc., Shiga, Japan). The expression vector was transformed into competent cells of *E. coli* BL21(DE3) by the heat shock method. After the inoculation and incubation of transformants on an LB agar plate containing 100 μg/mL ampicillin sodium, a single colony was picked and incubated in 3.5 mL of LB medium containing the same antibiotics. After 6 h of incubation, the cultured medium was added to 1 L of autoinduction medium containing 100 μg/mL ampicillin sodium, and the medium was cultured overnight at 25 ℃. After incubation, the cells were collected by centrifugation and resuspended in 10 mM Tris-HCl (pH 8.0). The cells were then sonicated for

15 min and centrifuged at 15000 × *g* for 20 min to separate cell debris. The supernatant was subjected to His-tag column chromatography using a HisTrap FF crude column and the purified α-SQase was collected and desalted into DPBS (pH 7.4). The purity of α-SQase was analyzed by SDS-PAGE. The concentration of the purified α-SQase was measured by determining the absorbance at 280 nm using a spectrophotometer (DS-11+, DeNovix, Delaware, United States of America).

2.12 Determination of enzymatic activity parameters of α-SQase

The activity of α-SQase for NPSQ was measured. NPSQ at various concentrations was reacted with 13.6 nM α-SQase in Dulbecco's phosphate buffered saline (DPBS) (pH 7.4) at 25 ℃, and the absorption (405 nm) of *p*-nitrophenol was measured using a plate reader (AD200, Beckman Coulter, California, United States of America) to obtain the reaction ratios. Measurements were performed within the time frame in which the reaction rate was considered constant. The kinetic constants were calculated from the Lineweaver-Burk plot.

2.13 Preparation of enzyme-antibody conjugate

SM $(PEG)_4$ [SM(PEG)4 (PEGylated SMCC crosslinker, Thermo Scientific, Massachusetts, United States of America] (10 mg/mL) in dimethyl sulfoxide was diluted ten times with buffer A (100 mM sodium phosphate, 150 mM NaCl, pH 7.2). The solution (34 μL) was added to anti-rabbit-IgG antibody (goat anti-rabbit IgG H&L, Abcam) (5 mg/mL) in buffer A (110 μL) and allowed to stand at room temperature for 1 h. Buffer B (100 mM sodium phosphate, 150 mM NaCl, pH 6.0) (2.36 mL) was added to the diluted solution, purified using a PD-10 column, and ultrafiltrated using an Amicon Ultra-0.5 device (NMWL: 50 K) to obtain anti-IgG antibody (5 mg/mL) in buffer A. 35 mM 2-iminothiolane in buffer C (100 mM sodium phosphate, 150 mM NaCl, 1 mM ethylenediaminetetraacetic acid (EDTA), pH 7.8) (4.7 μL) was added to 10 mg/mL α-SQase in buffer C (100 μL) ultrafiltered using an Amicon Ultra-0.5 device (NMWL: 50 K) and allowed to stand at room temperature for 1 h. Buffer C was added to the mixture to make the total volume 2.5 mL, purified using a PD-10 column, and ultrafiltrated using an Amicon Ultra-0.5 device (NMWL: 50 K) to obtain 5 mg/mL α-SQase in buffer A. The prepared antibody and enzyme solutions were mixed in a ratio of 1/2 and allowed to stand for 1 h. The reaction mixture was purified using a PD-10 column. The production of the enzyme-antibody conjugate was confirmed by SDS-PAGE.

2.14 Detection of antigen protein on cell surface

A549 ($CD44^+$) and HepG2 ($CD44^-$) cell suspensions (1×10^6 cells/mL) were prepared, and 100 μL of each was seeded in 96-well plates. After confirming cell adhesion under a microscope, the supernatant was removed. The primary antibody (anti-CD44 rabbit antibody, Abcam, Cambridge, United Kingdom of Great Britain and Northern Ireland) (1–10 μg/mL) in DMEM containing 2% FBS was immediately added to each cell type, which was incubated at 4 ℃ for 30

min. The cells were washed four times with DMEM containing 1% FBS. α-SQase modified antibody (1–10 μg/mL) in DMEM containing 2% FBS was added to each cell type, which was incubated at 4 ℃ for 30 min. The cells were washed five times with DMEM containing 1% FBS. NPSQ (1 mM) in DPBS (pH 7.4) was added, and the mixture was reacted at 37 ℃ for 5 h. The absorbance at 410 nm was measured using a plate reader (Infinite 200PRO M Plex, TECAN), and the change in absorbance was calculated.

2.15 Docking simulation of substate with α-SQase

The binding prediction of methylumbelliferyl α-sulfoquinovoside (MUSQ) and substrate 1 with α-SQase was performed. MUSQ and substrate 1 were docked into the docking model created using MOE (2022.02 Chemical Computing Group, Montreal, Canada). Their geometry restrain files can be obtained from eLBOW in phenix programs.[17] The MUSQ and substrate 1 were placed in the substrate-binding pocket of α-SQase based on the position of NPSQ. The docked substrates underwent energy minimization, with all protein atoms of α-SQase being fixed during the process.

2.16 Reactivity of substrate 1 toward α-SQase

Fluorescence spectra (FP-8601, JASCO, Tokyo, Japan) were acquired before and 1 h after adding 0.4 mM substrate 1 to 100 μg/mL (1.27 μM) α-SQase solution in DPBS (pH 7.4). The excitation wavelength was 365 nm. The reaction of substrate 1 or NPSQ with α-SQase was monitored as a function of time. First, 50 μM substrate 1 was reacted with 50 nM α-SQase in DPBS (pH 7.4) at 25 ℃, and the fluorescence intensity (ex. 365 nm, em. 445 nm) of 4-methylumbelliferone was monitored using a spectrofluorometer (FP-8601, JASCO). Then, 50 μM NPSQ was reacted with 50 nM α-SQase in DPBS (pH 7.4) at 25 ℃, and the absorption (410 nm) of *p*-nitrophenol was monitored with a UV–Vis–NIR spectrophotometer (V670iRM, JASCO).

3. Results and Discussion

3.1 Endogenous activity of α-SQase in human cell lines

According to the public databases UniProt and The Human Protein Atlas, no enzyme of α-SQase is reported in human. Thus far, the activity and crystal structure of two types of α-SQase (*E. coli* and *Agrobacterium tumefaciens*) with high homology were reported. They specifically react with the α-SQ substrate by recognizing the unique configuration of α-SQ.[11,12] Thus, we used here α-SQase derived from *E. coli*. First, we examined the absence of α-SQase activity in two types of human cell line (HeLa and JY25) using NPSQ. As shown in Fig. 2(a), the activity of phosphatase was observed in live cells and lysates. Moreover, β-galactosidase showed activity in cell lysates at pH 4.5, reflecting the presence of β-galactosidase in the lysosome.[7,8] In contrast, the activity of α-SQase was not detected in live cells or lysates, indicating the absence of its activity.

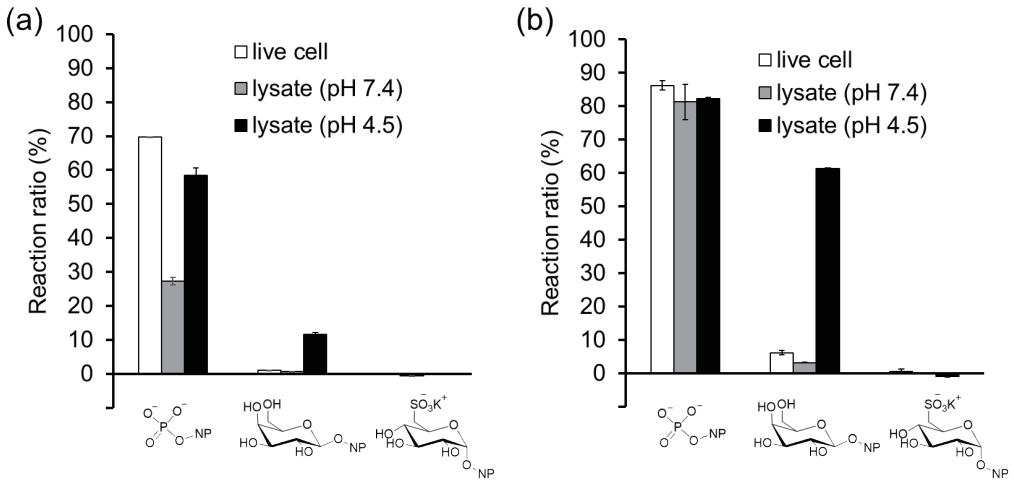

Fig. 2. (Color online) Reaction ratios of 3 mM substrates of phosphatase, *β*-galactosidase, and α-SQase added to live cells and lysates of (a) HeLa and (b) JY25 cells at 37 °C for 18 h. NP in the chemical structures indicates a *p*-nitrophenyl group. Data are means ± S.D, $n = 3$.

3.2 Cell ELISA

We examined the applicability of α-SQase from *E. coli* to cell ELISA [Fig. 3(a)], in which CD44 and NPSQ were selected as the target antigen and substrate, respectively. α-SQase was expressed from *E. coli* using a pDNA vector and purified using His-tag. Parameters of the enzymatic activity of the obtained α-SQase, which were determined using NPSQ, were as follows: $V_{max} = 2.0 \times 10^{-4}$ mM s^{-1}, $K_m = 0.32$ mM, and $k_{cat} = 16.0$ s^{-1}. These values were consistent with the reported values.[12] α-SQase was conjugated with secondary antibodies via a chemical crosslinker. As shown in Fig. 3(b), no signal was detected for HepG2 cells, which was CD44-negative. In contrast, a signal was detected for CD44$^+$ A549 cells, indicating the successful detection of CD44 on A549 cells. The background signal was detected for A549 cells with only α-SQase-conjugated secondary antibodies. Because the background signal was not detected for HepG2 cells, this background signal was likely derived from the nonspecific adsorption of the conjugates of α-SQase and secondary antibodies.

3.3 Design and synthesis of fluorescent substrate for α-SQase

Zhang *et al*. reported that the reaction between the MU substrate and α-SQase proceeds extremely slowly compared with that between NPSQ and α-SQase.[16] The k_{cat}/K_m value of the MU substrate is 10^4–10^5-fold smaller than that of NPSQ.[12,16] Because the natural substrates of α-SQase are diacylglyceride or glycerol derivatives of sulfoquinovose, the pocket of the substrate recognition site is expected to be too narrow to accommodate the bulky MU substrate. Figure 4(a) shows the crystal structure of the α-SQase mutant with NPSQ, in which one of the active residues, namely, D472, was substituted with Asn to diminish the activity of α-SQase. The active residues (D405, D472N) are located at the end of the deep, narrow pocket, where natural substrates with slender diacylglyceride and glycerol groups can be accommodated. However, in

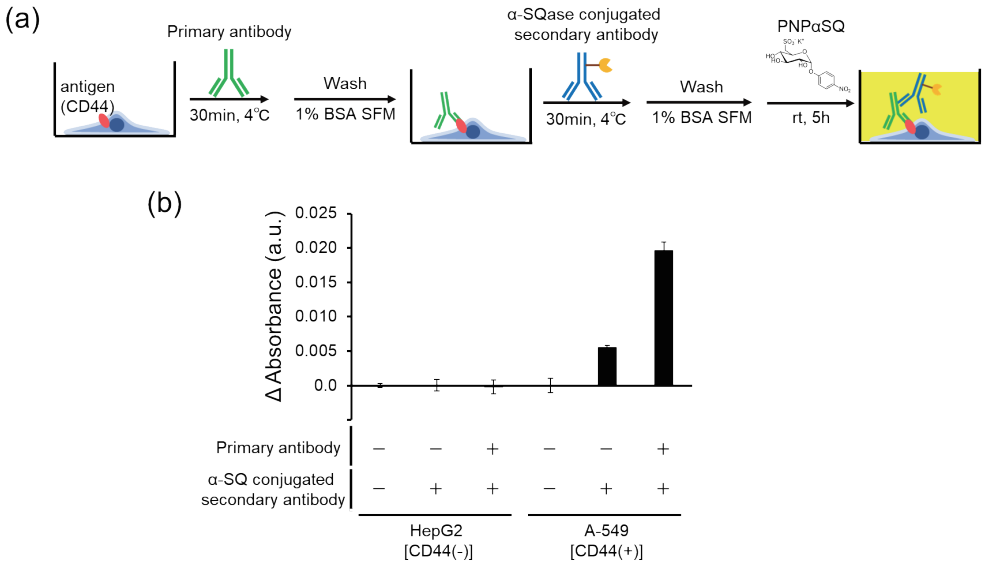

Fig. 3. (Color online) (a) Schematic drawing and (b) results of cell ELISA of CD44 on HepG2 [CD44(−)] and A549 [CD44(+)] cells using a secondary antibody conjugated with α-SQase. The reaction was conducted at 37 °C for 5 h, and 1.0 mM NPSQ was used for the detection. Data are means ± S.D, $n = 3$.

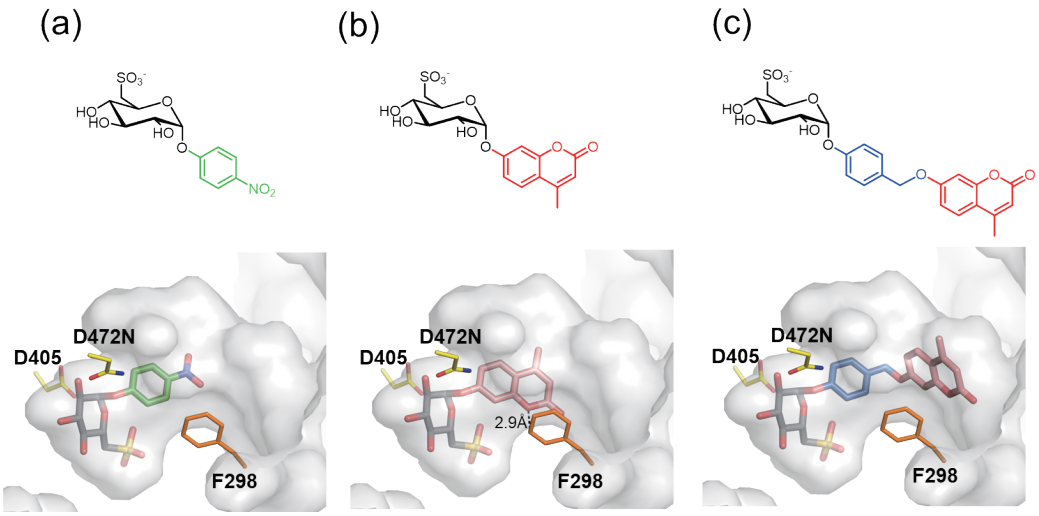

Fig. 4. (Color online) (a) Crystal structure of NPSQ with D472N-mutated α-SQase (PDB: 5OHS) and prediction of binding between (b) MUSQ and (c) substrate 1 with α-SQase.

the simulated model [Fig. 4(b)], the MU group of the substrate collides with F298 in the pocket, which impedes the enzymatic reaction. We designed a fluorescent substrate, denoted as substrate 1, in which the sulfoquinovose and MU groups are connected with a self-immolative hydroxy benzyl alcohol linker. This is the representative self-immolative linker, which is spontaneously removed after the hydrolysis of the ether bond of the phenol group.[18] As shown in Fig. 4(c), substrate 1 does not collide with the pocket, indicating its suitability as a substrate of α-SQase.

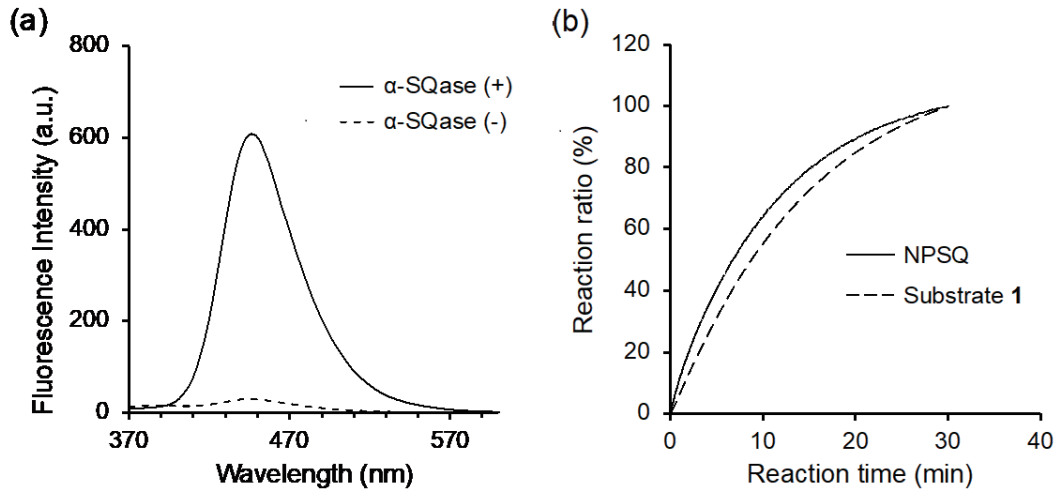

Fig. 5. (a) Fluorescence spectra of substrate 1 before and after its reaction with α-SQase. Substrate 1 (0.4 mM) was reacted with 100 μg/mL α-SQase in DPBS (pH 7.4) at 25 °C for 1 h. (b) Time course of the enzymatic reaction ratio of NPSQ and substrate 1 with α-SQase. The substrate (50 μM) was reacted with 50 nM α-SQase in DPBS (pH 7.4) at 25 °C.

Substate 1 was synthesized from acetyl β-D-glucopyranose in seven steps, as depicted in the scheme in Fig. 1. Figure 5(a) shows the fluorescence spectra of substrate 1 before and after the enzymatic reaction with α-SQase. The fluorescence of substrate 1 was negligible before the reaction but increased 19.5-fold after the reaction with α-SQase for 1 h. The hydroxy benzyl alcohol linker of substrate 1 contributes to quenching via the transfer of photoexcited electrons, thereby weakening the fluorescence. Figure 5(b) shows the fluorescence of substrate 1 as well as the absorbance of the *p*-nitrophenol substrate as a function of reaction time. The rates of the two reactions were similar, indicating that substrate 1 was a suitable substrate for the fluorescence monitoring of the activity of α-SQase.

5. Conclusions

In this research, we confirmed the absence of the enzymatic activity of α-SQase on the surface and inside of human cells. We showed that α-SQase was applicable to live-cell ELISA, which is not achievable by using conventional amplification enzymes. We succeeded in developing a fluorescent substrate for α-SQase by using a self-immolative linker, which enabled binding in the narrow, deep active pocket of α-SQase. α-SQase combined with its fluorescent substrate will be useful for the amplified detection of antigens in biological samples.

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