明らかとなり、共生培養が遺伝子資源の有効活用に重要であると考えた。
新規母核を有するグルコシダーゼ阻害剤を得るため、典型的な阻害剤である C7NA Family 化合物生合成遺伝子（acbC 遺伝子）を有していないと予想される放線菌 194 と 243a の共生培養液から阻害剤の単離・精製・構造決定を試みた。
その結果、C7NA Family 化合物ではない化合物 A 及び B を単離した。化合物 A は阻害活性本体ではなかったため 、現在化合物 B の構造解析及び阻害活性について検討を行っている。

F. 健康危険情報

特になし。

G. 業績

1) Design and Screening Strategies for α-Glucosidase Inhibitor Based on Enzymological Information. Wataru Hakamata, Masaaki Kurihara, Haruhiro Okuda, Toshiyuki Nishio, Tadatake Oku, *Current Topics in Medicinal Chemistry*, 9 (1), 3-12 (2009).

2) Crystallization and structural analysis of cytochrome *c6* from the diatom *Phaeodactylum tricornutum* at 1.5 Å resolution. Hideharu Akazaki, Fumihiro Kawai, Masahiro Hosokawa, Toshiyuki Hama, Hirotaka Chida, Takako Hirano, B. K. Lim, N Sakurai, Wataru Hakamata, Toshiyuki Nishio, sam-Yong Park, Tadatake Oku, *Bioscience, Biotechnology, and Biochemistry*, 73, 189-191 (2009).

3) Purification, characterization, and cloning of *Vibrio parahaemolyticus* chitinolytic enzymes and application to oligosaccharide production. Kazunari Kadokura, Yusuke Sakamoto, Akiko Rokutani, Takanori Ikegami, Takako Hirano, Mahiro Yamamoto, Kaori Saito, Wataru Hakamata, Shiro Itoi, Haruo Sugita, Tadatake Oku, Toshiyuki Nishio, *Journal of Applied Glycoscience*, 55, 157-164, (2008).

4) Cloning, expression and purification of cytochrome *c6* from the brown alga *Hizikia fusiformis* and complete X-ray diffraction analysis of the structure. Hideharu Akazaki, Fumihiro Kawai, Hirotaka Chida, Yuichirou Matsumoto, Mao Hirayama, Ken Hoshikawa, Satoru Unzai, Wataru Hakamata, Toshiyuki Nishio, sam-Yong Park, Tadatake Oku, *Acta Crystallographica F.*, 64, 674-680, (2008).

5) Physicochemical properties of diheme cytochrome *c4* of unknown function from *Vibrio parahaemolyticus* strain RIMD2210633. Hideharu Akazaki, Yoshio Futami, Naoya Shibayama, Ikuko Shirasaki, Harumi Nakade, Hirotaka Chida, Wataru Hakamata, sam-Yong Park, Toshiyuki Nishio, Tadatake Oku, *Bioscience Biotechnology and Biochemistry*, 72, 2791-2794 (2008).

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H. 知的財産権の出願・登録状況

現在、本研究で得られた細胞レベルで

抗 HIV 活性が認められた化合物について、日本大学・国立医薬品食品衛生研究所・国立感染症研究所の知財部および職務発明委員会を通して、特許の出願手続きを行っている。

別紙４

研究成果の刊行に関する一覧表

書籍

著者氏名	論文タイトル名	書籍全体の編集者名	書籍名	出版社名	出版地	出版年	ﾍﾟｰｼﾞ
本年度は該当なし							

雑誌

発表者氏名	論文タイトル名	発表誌名	巻号	ページ	出版年
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6. Physicochemical properties of diheme cytochrome *c4* of unknown function from *Vibrio parahaemolyticus* strain RIMD2210633. Hideharu Akazaki, Yoshio Futami, Naoya Shibayama, Ikuko Shirasaki, Harumi Nakade, Hirotaka Chida, Wataru Hakamata, sam-Yong Park, Toshiyuki Nishio, Tadatake Oku, *Bioscience Biotechnology and Biochemistry*, 72, 2791-2794 (2008).

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11. Computational Study on Secondary Structure of Oligopeptides Containing α,α-Disubstituted α-Amino Acids; M. Kurihara, Y. Sato, F. Kaneko, H. Okuda, M. Nagano, Y. Demizu, M. Doi, M. Tanaka, H. Suemune; *Peptide Science 2007*, 137-138, (2008).

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Design and Screening Strategies for α-Glucosidase Inhibitors Based on Enzymological Information

Wataru Hakamata[1],*, Masaaki Kurihara[2], Haruhiro Okuda[2], Toshiyuki Nishio[1] and Tadatake Oku[1]

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Abstract: α-glucosidase inhibitors are marketed as therapeutic drugs for diabetes that act through the inhibition of carbohydrate metabolism. Inhibitors of the α-glucosidases that are involved in the biosynthesis of *N*-linked oligosaccharide chains have been reported to have antitumor, antiviral, and apoptosis-inducing activities, and some have been used clinically. α-Glucosidase inhibitors have interesting biological activities, and their design, synthesis, and screening are being actively performed. In quite a few reports, however, α-glucosidases with different origins than the target α-glucosidases, have been used to evaluate inhibitory activities. There might be confusion regarding the naming of α-glucosidases. For example, the term α-glucosidase is sometimes used as a generic name for α-glucoside hydrolases. Moreover, IUBMB recommends the use of "α-glucosidase" (EC 3.2.1.20) for exo-α-1,4-glucosidases, which are further classified into four families based on amino acid sequence similarities. Accordingly, substrate specificity and susceptibility to inhibitors varies markedly among enzymes in the IUBMB α-glucosidases. The design and screening of inhibitors without consideration of these differences is not efficient. For the development of a practical inhibitor that is operational in cells, HTS using the target α-glucosidase and the computer-aided design of inhibitors based on enzymatic information concerning the same α-glucosidase are essential.

Keywords: α-glucosidase, substrate specificity, inhibitor, HTS, virtual screening, *in silico*, structure based drug design.

INTRODUCTION

Inhibitors of small intestinal α-glucoside hydrolases were revealed to improve postprandial hyperglycemia in the 1970s and were approved as therapeutic drugs for diabetes in the 1990s. Recently, sugar chains have been attracting attention as a third class of biopolymer following nucleic acids and proteins, and now the elucidation of sugar chain functions is indispensable in postgenomic studies. This is because glycoproteins and glycolipids play important roles in many biotic phenomena, such as embryogenesis, differentiation, cancer, infection, inflammation, aging, reproduction, and regeneration. Accordingly, the *N*-linked oligosaccharide processing enzymes, e.g., glycosidases or glycosyltransferases have emerged as new molecular targets in drug development studies. Inhibitors of these enzymes are seen as potential drug seeds as well as tools for the elucidation of biotic phenomena. In this review, we survey the classification of the α-glucoside hydrolases involved in carbohydrate metabolism and *N*-linked oligosaccharide biosynthesis, detailing differences in their nomenclature, reaction mechanisms, and substrate specificity. In addition, the construction of a HTS system for α-glucosidase inhibitors and *in silico* inhibitor design and screening based on the three-dimensional structures of α-glucosidases are described.

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Classification of α-Glucosidases and Related Enzymes

The CAZy database [1] classifies carbohydrate-related enzymes based on amino acid sequence similarities [2-5]. In CAZy, most α-glucosidases (EC 3.2.1.20) are classified into GH13 and GH31, and the others are classified into GH4 and GH97. ER processing α-glucosidase I (EC 3.2.1.106, official name: Mannosyl-oligosaccharide glucosidase) and ER processing α-glucosidase II (EC 3.2.1.84, official name: Glucan 1,3-α-glucosidase) are known as *N*-linked oligosaccharide processing enzymes and are classified into GH63 and GH31, respectively. These enzymes are exo-type glycosidases that hydrolyze α-D-glucopyranoside linkages. During the hydrolysis of α-glucosidases, the GH13 and GH31 α-glucosidases release α-D-glucopyranose with retention of the configuration in the anomeric position [6,7], and processing α-glucosidase I releases β-D-glucopyranose and causes inversion of the configuration [8]. The anomeric configuration of D-glucopyranose released by processing α-glucosidase II has not been identified. Glucoamylase (EC 3.2.1.3) is part of a group of enzymes that hydrolyze α-glucopyranosides from the non-reducing end in an exo-type manner and release β-D-glucopyranose. Its reaction mechanism is different from that of α-glucosidases (EC 3.2.1.20) and it should be strictly distinguished from them. The CAZy classificationof α-glucosidases and related enzymes, reaction types, and the anomeric configurations of the products produced are summarized in Table **1**. Furthermore, commercially available α-glucosidases are classified according to their GH number in CAZy as shown in Table **2**.

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Table 1. Classification of α-Glucosidase and Related Enzymes

Official Name	E.C. Number	Glycoside Hydrolase Family Number	Product	Anomeric Configuration
α-Glucosidase	3.2.1.20	4, 13, 31, 97	α-Glucose	Retain
Mannosyl-oligosaccharide glucosidase	3.2.1.106	63	β-Glucose	Inversion
Glucan 1,3-α-glucosidase	3.2.1.84	31	Unknown	Unknown
Oligo-1,6-glucosidase	3.2.1.10	4, 13, 31	α-Glucose	Retain
Sucrose α-glucosidase	3.2.1.48	31	α-Glucose	Retain
β-Fructofuranosidase	3.2.1.26	32, 68, 100	α-Glucose	Retain
Glucan 1,4-α-glucosidase	3.2.1.3	15	β-Glucose	Inversion

Table 2. Glycoside Hydrolase Family Number of Commercially Available α-Glucosidases

Origin	Glycoside Hydrolase Family Number	Supplier
S. cerevisiae	13	Sigma
B. stearothermophilus	13	Sigma
A. niger	31	Megazyme
Rice	31	Sigma

α-Glucosidases (EC 3.2.1.20) are also known by other names: maltase, acid maltase, glucoinvertase, glucosidosucrase, lysosomal α-glucosidase, and maltase-glucoamylase. Enzymes corresponding to α-glucosidase include oligo-1,6-glucosidase (EC 3.2.1.10) and sucrose α-glucosidase (EC 3.2.1.48), and also these are called isomaltase and sucrase, respectively. α-Glucosidase and related enzymes have various other names. These similar names sometimes cause confusion in the interpretation and evaluation of research results. Official names and alternative names of α-glucosidase and related enzymes are summarized in Table **3**.

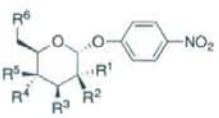
Acquisition of the Enzymological Information of α-Glucosidases for Inhibitor Design using Synthetic Small Molecules

In some reports, α-glucoside hydrolases, which include all the enzymes that cleave α-D-glucopyranoside bonds in an exo-type manner and release glucose, and the specific α-glucosidases (EC 3.2.1.20) are mentioned as if they were identical, and inhibitor designs seem to be based on the enzymes different from those used in the assays. For example, some α-glucosidase inhibitors were designed and synthesized for use as antiviral drugs that would block *N*-linked oligosaccharide processing in host cells, but these were evaluated using the α-glucosidase from yeast (*S. cerevisiae*) and/or other sources. The yeast α-glucosidase belongs to GH13, whereas the targets of antiviral drugs are processing α-glucosidases I and II. These evaluations are inadequate for discovery of processing α-glucosidase I and II inhibitors, even if the inhibitors a thoroughly designed. It is not unusual that strong yeast α-glucosidase inhibitors often exhibit few effects toward mammalian cells, though this may also be related to the transferability of the inhibitors into cells. In other words, a low inhibitory activity against yeast α-glucosidase does not necessarily translate into low activity against processing α-glucosidase I and II. For example, a typical α-glucosidase inhibitor, 1-deoxynojirimycin, inhibits yeast α-glucosidases (IC_{50} = 12.6 μM [9]) *in vitro*. However, 1-deoxynojirimycin has very weak effects against BVDV, which serves as a model organism for HCV at the cellular level [10]. The potent α-glucosidase inhibitor kojibiose type pseudodisaccharide and its derivatives show low IC_{50} values (120 nM - 3.1 μM) and have no effect against processing glucosidases [11].

We have examined the substrate specificity of α-glycosidases for inhibitor design using small synthetic molecules [12-21], some of which are summarized in Fig. (**1**). Among the α-glucosidases, the GH31 α-glucosidases (from rice, *A. niger*, flint corn, and sugar beet) and processing α-glucosidase II (from rat liver microsomes) possessed α-2-deoxy-glucosidase activity, and this activity was about twice as high as their the α-glucosidase activity. The C-2 hydroxyl groups of the glycons are not essential for the hydrolysis action of the GH31 α-glucosidases and processing α-glucosidase II, while GH13 α-glucosidases (*S. cerevisiae*, *B. stearothermophilus*, and honeybee isozyme I, II, and III) all require the hydroxyl groups of the glycons for their hydrolyzing activity. α-Mannosidases from jack beans

Table 3. Official Name and Alternative Name of α-Glucosidase and Related Enzymes

Official Name	E.C. Number	Alternative Name (s)
α-Glucosidase	3.2.1.20	Maltase, Acid maltase, Glucoinvertase, Glucosidosucrase Lysosomal α-glucosidase, Maltase-glucoamylase
Mannosyl-oligosaccharide glucosidase	3.2.1.106	Processing α-glucosidase I, Glucosidase I
Glucan 1,3-α-glucosidase	3.2.1.84	Processing α-glucosidase II, Glucosidase II, Exo-1,3-α-glucanase
Oligo-1,6-glucosidase	3.2.1.10	Isomaltase, Oligosaccharide α-1,6-glucosidase Sucrase-isomaltase
Sucrose α-glucosidase	3.2.1.48	Sucrase, Sucrase-isomaltase, Sucrose α-glucohydrolase
β-Fructofuranosidase	3.2.1.26	β-Fructosidase, Invertase, Saccharase
Glucan 1,4-α-glucosidase	3.2.1.3	Glucoamylase, 1,4-α-D-Glucan glucohydrolase Amyloglucosidase, Exo-1,4-α-glucosidase γ-Amylase, Lysosomal α-glucosidase



Glucopyranoside derivatives
1: R^1=OH, R^2=H, R^3=OH, R^4=OH, R^5=H, R^6=OH (PNP Glc)
2: R^1=R^2=H, R^3=OH, R^4=OH, R^5=H, R^6=OH (PNP 2D Glc)
3: R^1=OH, R^2=R^3=H, R^4=OH, R^5=H, R^6=OH (PNP 3D Glc)
4: R^1=OH, R^2=H, R^3=OH, R^4=R^5=H, R^6=OH (PNP 4 D Glc)
5: R^1=OH, R^2=H, R^3=OH, R^4=OH, R^5=R^6=H (PNP 6D Glc)

Mannopyranoside derivatives
6: R^1=H, R^2=OH, R^3=OH, R^4=OH, R^5=H, R^6=OH (PNP Man)
2: R^1=R^2=H, R^3=OH, R^4=OH, R^5=H, R^6=OH (PNP 2D Man)
7: R^1=H, R^2=OH, R^3=H, R^4=OH, R^5=H, R^6=OH (PNP 3D Man)
8: R^1=H, R^2=OH, R^3=OH, R^4=R^5=H, R^6=OH (PNP 4 D Man)
9: R^1=H, R^2=OH, R^3=OH, R^4=OH, R^5=R^6=H (PNP 6D Man)

Galactopyranoside derivatives
10: R^1=OH, R^2=H, R^3=OH, R^4=H, R^5=OH, R^6=OH (PNP Gal)
11: R^1=R^2=H, R^3=OH, R^4=H, R^5=OH, R^6=OH (PNP 2D Gal)
12: R^1=OH, R^2=R^3=R^4=H, R^5=OH, R^6=OH (PNP 3D Gal)
4: R^1=OH, R^2=H, R^3=OH, R^4=R^5=H, R^6=OH (PNP 4 D Gal)
13: R^1=OH, R^2=H, R^3=OH, R^4=H, R^5=OH, R^6=H (PNP 6D Gal)

Fig. (1). Synthetic Small molecules as substrates for profiling the molecular recognition of α-glucosidase.

and almonds had α-6-deoxy-mannosidase activity levels equivalent to their α-mannosidase activitiy levels. Among the α-galactosidases, α-galactosidase from green coffee beans and *M. vinacea* had weak α-2-deoxy-galactosidase and α-6-deoxy-galactosidase activity, and *A. niger* α-galactosidase showed α-2-deoxy-galactosidase activity with a strength twice as great as its α-galactosidase activity. These results are summarized in Table **4**. The specificities for the deoxyglycons varied markedly throughout the different glycosidase families, while each family retained its original glycon specificity, strongly indicating that the major differences between the α-glucosidase family are due to the residues in the glycon recognition sites (subsite -1) in Fig. (**2**).

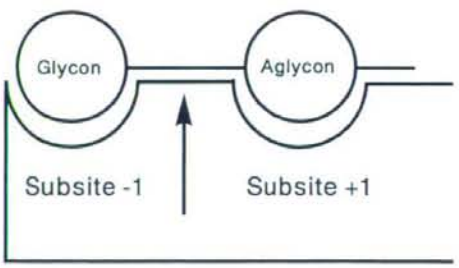


Fig. (2). Schematic diagram of substrates bound to the α-glucosidase subsite. Hydrolysis, shown by an arrow, takes place between subsites -1 and +1.

α-Glucosidases as Molecular Targets for Drug Discovery

Therapeutic drugs for type 2 diabetes, such as acarbose (Bayer), miglitol (Bayer), and voglibose (Takeda) in Fig. (**3**), inhibit small intestinal α-glucoside hydrolases (gluco-amylase (EC 3.2.1.3), sucrase (EC 3.2.1.48), and α-glucosidase (EC 3.2.1.20) [22]. These drugs only affect postprandial glucose levels and do so by competitively inhibiting the binding of oligosaccharides to the α-glucoside hydrolases. These enzymes cleave oligosaccharides to monosaccharides, which can then be absorbed. Thus, when taken with the first bite of food, these agents delay the absorption of carbohydrates. These are the first-line drugs for type 2 diabetes, and their importance is very high [23]. They are particularly important because the cost of diabetes to the United States healthcare system amounts to $100 billion in direct and indirect expenditure annually [24].

Table 4. Relative Rates of Hydrolysis of Monodeoxy Substrates Using α-Glycosidases [9-11, 13-14]

Enzyme / Substrate	Relative Rate of Hydrolysis (%)				
	PNP Glc (1)	PNP 2D Glc (2)	PNP 3D Glc (3)	PNP 4D Glc (4)	PNP 6D Glc (5)
α-Glucosidase					
S. cerevisiae (GH13)	100	–	–	–	–
B. stearothermophilus (GH13)	100	–	–	–	–
Honeybee I (GH13)	100	–	–	–	–
Honeybee II (GH13)	100	–	–	–	–
Honeybee III (GH13)	100	–	–	–	–
Rice (GH31)	100	175	–	–	–
Sugar beet (GH31)	100	244	–	–	–
Flint corn (GH31)	100	231	3.7	–	–
A. niger (GH31)	100	259	11.9	–	–
Processing α-glucosidase II Rat liver microsome (GH31)	100	189	–	–	–
Enzyme / Substrate	Relative rate of hydrolysis (%)				
	PNP Man (**6**)	PNP 2D Man (**2**)	PNP 3D Man (**7**)	PNP 4D Man (**8**)	PNP 6D Man (**9**)
α-Mannosidase					
Canavalia ensiformis (Jack bean, GH38)	100	–	–	–	92.2
Prunus dulcis (Almond)	100	–	–	–	118
Enzyme / Substrate	Relative rate of hydrolysis (%)				
	PNP Gal (**10**)	PNP 2D Gal (**11**)	PNP 3D Gal (**12**)	PNP 4D Gal (**4**)	PNP 6D Gal (**13**)
α-Galactosidase					
Green coffee bean	100	15.0	–	–	3.3
M. vinacea (GH27)	100	19.0	–	–	8.8
A. niger (GH27)	100	230	–	–	–

Relative rate of hydrolysis is expressed by comparison with the amount of *p*-nitrophenol released from PNP Glc (**1**), PNP Man(**6**), and PNP Gal (**10**), respectively which is taken as 100%. – : Hydrolytic activity was not detected.

Inhibitors of *N*-linked oligosaccharide processing α-glucosidases I and II have various useful physiological and biological activities. *N*-Linked oligosaccharides are attached to many nascent proteins as posttranslational modifications. The formation of the *N*-linked oligosaccharide chains in the ER starts with the attachment of $Glc_3Man_9GlcNAc_2$ to a nascent protein and removal of the glucose residues by processing α-glucosidases I and II [25,26]. The involvement of the processing of glucose and mannose residues in quality control [27,31] and ERAD [32-35] of glycoproteins in the ER has recently been clarified. Inhibitors of processing α-glucosidase I and II and the other *N*-linked oligosaccharide chain processing enzymes disturb protein folding and intracellular transport by inhibiting *N*-linked oligosaccharide chain formation [36,37] causing antitumor activities [38,44], antiviral activities (HIV [45-46], HCV [47-48], HBV [49], influenza [50], SARS [51,52], etc. [53]), and apoptosis induction [54,55]. The destruction caused by ERAD is considered to be a major cause of neurodegenerative diseases such as Alzheimer's disease and Parkinson's disease [56], and *N*-linked oligosaccharide chain processing enzymes have been attracting attention as molecular targets for new drug development [57,59].

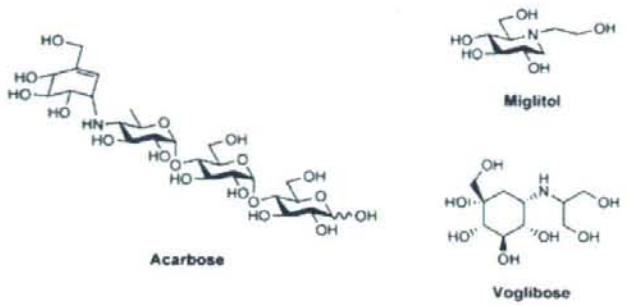


Fig. (3). Chemical structures of drugs for type 2 diabetes.

Characteristics of α-Glucosidase Inhibitors

Forty years have passed since the most renowned glucosidase inhibitor, nojirimycin, was first reported in 1966 [59,62]. Since then, an enormous number of glucosidase inhibitors have been discovered, synthesized, and have had their inhibitory activities investigated [64-73].

The reported α-glucosidase inhibitors have some of the following characteristics: (1) sugar (substrate)-mimic structures, (2) the ability to form ionic bonds with nucleophilically catalyzing residues, (3) transition-state-like structures, (4) the ability to form hydrogen bonds with catalytic acid residues, (5) the ability to make ionic and hydrophobic interactions at sites other than the active site, and (6) the ability to form covalent bonds with enzymes through an epoxy or aziridine group. Archetypal examples are summarized in Fig. (**4**). These model structures are reported to be potent inhibitors that combine the above-mentioned (1)-(6) features. The target of these α-glucosidase inhibitors is the glycon binding subsite (subsite -1) of α-glucosidases. However, non-sugar-mimicking α-glucosidase inhibitors have recently been reported (Fig. (**5**)) [74-76] and these inhibitors might bind to the aglycon binding site or elsewhere.

HTS of α-Glucosidase Inhibitors

The major target enzymes of the α-glucosidase in drug development are the small intestinal α-glucosidases involved in the carbohydrate metabolic system and the processing α-glucosidases I and II involved in *N*-linked oligosaccharide chain processing. To obtain inhibitor leads, HTS toward the glycosidases has been aggressively carried out and reported in recent years [77,79]. To establish practical HTS methods for these enzymes, the use of the target α-glucosidase and substrate selection are important. In view of the advances in molecular biology, it is not difficult to prepare active enzymes by expressing human-derived target enzymes. The substrates should be selected carefully with consideration of the markedly different specificities of the target enzymes [80,87]. Generally, GH13 α-glucosidases exhibit high hydrolytic activity toward monosaccharide substrates, but GH31 α-glucosidases and processing α-glucosidase I and II show higher hydrolytic activity toward oligosaccharides than monosaccharides. α-Glucopyranosides with a chromophores

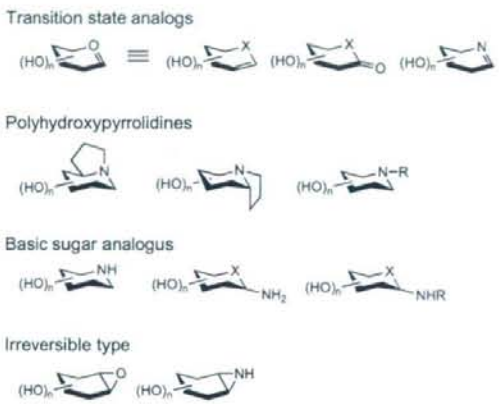
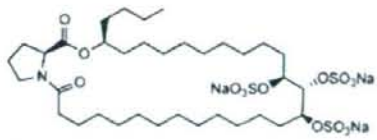
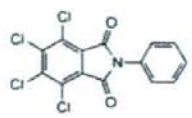


Fig. (4). Classification of typical α-glucosidase inhibitors [64].

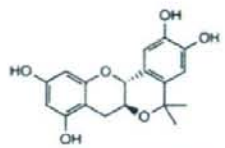
or fluorophores conjugated at the anomeric positions are widely used as substrates. Their activities can be measured simply and sensitively using these substrates; however, these substrates are still insufficient because large amounts of enzyme are necessary to measure GH31 α-glucosidases and processing α-glucosidase II activity owing to their low specificity toward mono-saccharides [88]. The reported α-glucosidase inhibition activity measurements have paid little attention to the amounts or activity units of the enzymes. The activity of the GH31 α-glucosidases was measured using more than ten times the amount of enzyme used for measuring the activity of the GH13 α-glucosidases. We developed PNP α-D-2-deoxy-glucopyranoside (**2**) as a highly specific substrate for GH31 α-glucosidases and processing α-glucosidase II and found that the activity of 2 as twice as high as that of PNP α-D-glucopyranoside (**1**) [14]. We are developing a chromophoric monosaccharide substrate for processing α-glucosidase II with a higher specific activity. A 20% reduction in the amount of enzyme required has been accomplished by this method. Although processing α-glucosidase I is an important molecular target for drug development to the best of our knowledge, there have been no previous reports on the development of a substrate for processing α-glucosidase I for use in HTS. Unlike GH13 α-



Penarolide sulfate A_1. IC_{50} = 1.2 μg / mL. [73]



CP0P. IC_{50} = 2.6 μM. [74]



PC1. IC_{50} = 0.7 μM. [75]

Fig. (5). Chemical structures of non-sugar-mimicking α-glucosidase inhibitors [73-75].

glucosidase, processing α-glucosidase I does not use monosaccharide derivatives as its substrate, but instead is inhibited by monosaccharide substrates [14, 89]. A simple processing α-glucosidase I assay method has been reported [14, 90,91], but it is not appropriate for HTS because it requires impractical materials such as a virus as the substrate and a radioisotope for the reaction tracing. We analyzed the molecular recognition of subsite -1 of processing α-glucosidase I using synthetic small molecules [14], but have not been able to develop a substrate adequate for HTS. Development of HTS for processing α-glucosidase I is an important issue and urgent resolution is needed.

In silico Techniques used in Computer Aided Molecular Design and Virtual Screening of α-Glucosidase Inhibitors

Computer-aided molecular design, "*in silico* molecular design", and computer ligand screening "*in silico* virtual screening", have recently become important with the rapid increase in the information available concerning three-dimensional protein structures and the development of high-speed computer-processing technologies. *In silico* molecular design is essential for the structural optimization of the active compounds obtained by HTS. *In silico* virtual screening of real (commercial and house) and virtual libraries and the molecular design of compounds that fit the ligand binding domain of a target protein have been effectively used to develop inhibitors [92,94].

In 2004-2006, the three-dimensional structures of the GH31 α-glucosidases *S. solfataricus* α-glucosidase (PDB ID: 2G3M and 2G3N) [95] and GH31 α-glycosidase YicI (PDB ID: 1XSI, 1XSJ, and 1XSK) [96] as well as *E. coli* α-xylosidase (PDB ID: 1WE5 [97] and 2F2H [98]) were solved. An *E. coli* α-xylosidase with an α-glucosidase activity was prepared by a mutation of its active site [99, 100]. The structure of *S. solfataricus* α-glucosidase shows differences from that of the other GH31 α-glucosidase, the *E. coli* α-xylosidase, although the enzymes have similar $(\beta/\alpha)_8$ barrels. For the main frame of GH13 α-glucosidase *in silico*, the structure of *B. cereus* oligo-1,6-glucosidase (EC. 3.2.1.10, isomaltase, PDB ID: 1UOK) [101] has been generally used. Fig. (**6**) shows the three-dimensional structures of a GH13 α-glucosidase (1UOK) and two GH31 α-glucosidases (2G3N and 1WE5). The three-dimensional structures of processing α-glucosidases I and II have not yet been reported.

An *in silico* molecular design of a glycosidase inhibitor is carried out by calculating the minimum interaction energies between a ligand and the active site of an enzyme. The most successful case of this method was performed for a marketed anti-influenza agent, Zanamivir, which acts through the inhibition of an influenza virus neuraminidase [103,107]. Clarification of the three-dimensional structure of enzymes enables the evaluation of existing inhibitors and the theoretical design of novel inhibitors. Before *in silico* technologies, many articles discussed the necessity of the SBDD approach, and their importance has increased. For the *in silico* design of α-glucosidase inhibitors using the three-dimensional structures of α-glucosidases, the following methods are applicable: (1) Structural optimization of existing inhibitors, (2) screening of real and virtual compound libraries by docking simulations, and (3) *de novo* design of inhibitors that bind to enzyme active sites, and several papers have reported virtual screening of α-glucosidase inhibitors [108-110]. Methods (2) and (3) will allow for the discovery of structurally novel inhibitors while such inhibitors have been previously discovered mainly from natural occurring substances. In the near future, computer simulations aimed at SBDD will produce excellent inhibitors in the virtual world. One of the current difficulties with *in silico* molecular design resides in the algorithm for the induced-fit phenomena of enzymes. Development of the algorithm is underway, and the design of inhibitors in consideration of induced-fit will be possible in the near future [111,112].

Summary and Discussion

New pieces of information on the functions of oligosaccharides in glycoproteins and glycolipids are reported frequently, expanding the potential physiological roles of α-

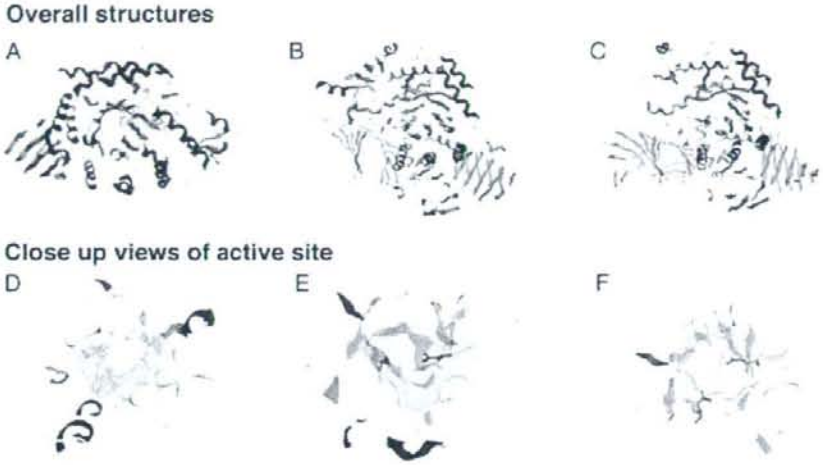


Fig. (6). Crystal structures of *B. cereus* oligo-1,6-glucosidase (A and D, 1UOK; belonging to GH13), *S. solfataricus* α-glucosidase (B and E, monomer structure, 2G3N; belonging to GH31), and *E. coli* α-xylosidase (C and F, monomer structure, 1WE5; belonging to GH31). The $(\beta/\alpha)_8$ barrels are shown using ribbon representations with green β-strands, red α-helices, and white coil segments. The cavities of active sites are represented in gray. The catalytic nucleophile and acid/base residues are shown as stick models in red. The figure was produced using Chemical Computing Inc's Molecular Operating Environment (MOE) version 2007.0901 [102].

glucosidase inhibitors. The importance of *N*-linked oligosaccharide processing enzyme inhibitors has been increasing not only as molecular targets in the development of new drugs but also as tools for the elucidation of biotic phenomena.

Many reports concerning the design, synthesis, and screening of α-glucosidase inhibitors have paid little attention to the classification and multiple naming of α-glucosidases or the differences in the molecular recognitions and three-dimensional structures of α-glucosidases. However, precise molecular designs based on the three-dimensional structures of α-glucosidases require close observation of molecule-enzyme interactions. No assay systems meeting HTS demands have been established for processing α-glucosidase I and II or the other *N*-linked oligosaccharide processing enzymes despite the increasing importance of these enzymes and their inhibitors from chemical biological viewpoints. The establishment of a HTS system for inhibitory activity evaluations using target enzymes and suitable substrates will provide abundant information on the structure-activity relationship, and *in silico* molecular design and screening based on this information and enzyme three-dimensional structural information will lead to the discovery of compounds with novel chemical structures, inhibitory mechanisms, and inhibitory activities. Furthermore, the elucidation of biotic phenomena and the development of novel drugs using these inhibitors is expected.

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ABBREVIATIONS

HTS	=	High throughput screening
IUBMB	=	International Union of Biochemistry and Molecular Biology
CAZy	=	Carbohydrate-active enzymes
GH	=	Glycoside hydrolase family
ER	=	Endoplasmic reticulum
BVDV	=	Bovine viral diarrhea virus
HCV and HBV	=	Hepatitis C and B virus
HIV	=	Human immunodeficiency virus
SARS	=	Severe acute respiratory syndrome
S. cerevisiae	=	*Saccharomyces cerevisiae*
A.	=	*Aspergillus*
B.	=	*Bacillus*
M.	=	*Mortirella*
S. solfataricus	=	*Sulfolobu solfataricus*
E.	=	*Escherichia*
ERAD	=	Endoplasmic reticulum-associated degradation
PNP	=	*p*-nitrophenyl
SBDD	=	Structure based drug design

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Note

Crystallization and Structural Analysis of Cytochrome c_6 from the Diatom *Phaeodactylum tricornutum* at 1.5 Å Resolution

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We determined for the first time the crystal structure of diatom cytochrome c_6 from *Phaeodactylum tricornutum* at 1.5 Å resolution. The overall structure of the protein was classified as a class I *c*-type cytochrome. The physicochemical properties of the protein were examined by denaturation with guanidine hydrochloride and urea, and compared with those of other algal cytochrome c_6.

Key words: cytochrome c_6; crystal structure; diatom; physicochemical property; structural stability

It is thought that diatoms acquired their chloroplasts and photosynthetic proteins *via* secondary endosymbiosis involving a primitive red algal endosymbiont and a non-photosynthetic eukaryote host.[1,2] The chlorophylls and photosynthetic electron carriers are different among cyanobacteria, diatoms, and green, red, and brown algae. In the case of green algae that contain chlorophyll *b* and cyanobacteria, a heme-Fe protein cytochrome (cyt) c_6 and a copper protein plastocyanin (PC) function as electron carriers between cyt *f*, which is part of the membrane-embedded cytochrome b_6f complex, and the P700 reaction center of photosystem I. It is widely believed that PC is not present in chlorophyll-*b*-non-containing algae such as red, and brown algae and diatoms, and that only cyt c_6 acts as an electron carrier in these organisms. But recent study has shown for the first time that a marine diatom, *Thalassiosira oceanica*, contains a PC.[3] To date, diatom PC has been confirmed only in marine diatom *T. oceanica*, and the closely related coastal species *Thalassiosira weissflogii* does not contain this copper protein.

Cyt c_6 is a high-potential, soluble, low-spin heme protein. Although the physicochemical properties and cDNA sequence of the protein from the diatom *Phaeodactylum tricornutum* have been examined,[4,5] the tertiary structure of diatom cyt c_6 remains unresolved. In this study, we determined the crystal structure and physicochemical properties of diatom cyt c_6, and compared them with those of other algal and cyanobacterial cyts c_6.

P. tricornutum (UTEX no. 646) was grown at 20 °C for 8 d under a 12 h light/12 h dark cycle at 60 $\mu E\,m^{-2}\,S^{-1}$ with fluorescent lamps in PES medium supplemented with 100 mg/l of $NaNO_3$, 0.1 mg/l of thiamine HCl, 1.0 µg/l of vitamin B_{12}, 7.0 mg/l of $Fe(NH_4)_2{\cdot}6H_2O$, 2.0 mg/l of H_2BO_3, 3.0 mg/l of Na_2-EDTA, 0.4 mg/l of $MnCl_2$, 0.01 mg/l of $CaCl_2$, 40 µg/l of $ZnCl_2$, 0.1 mg/l of $FeCl_2$, and 90 mg/l of $Na_2SiO_3{\cdot}9H_2O$. cDNA cloning of *P. tricornutum* cyt c_6 (Ptc6) was carried out by previous methods.[5] The mature Ptc6 sequence was amplified using a forward primer (5′-AGCCATGGGGGACGTCGGTGCTGGTG-AGC-3′), corresponding to the codons for the amino acid residues of the cyt c_6 N-terminal region, and a reverse primer (5′-GCGGATCCTTACTCCCATCCGG-CTTCAGCG-3′), corresponding to the codons for the amino acid residues of the C-terminal region. Vector construction, overproduction in *Escherichia coli*, and purification of recombinant Ptc6 were performed as described methods.[6] The degree of purity was confirmed by tricine sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

The UV/visible spectra and redox titration of the purified recombinant Ptc6 were measured according to previous methods.[7] The UV/vis absorption spectra of reduced and oxidized Ptc6 are shown in Fig. 1A. In the reduced form, the α-, β-, γ (soret)-, and δ-absorption maxima peaks appeared at 553.0 nm, 523.0 nm, 417.0 nm, and 316.0 nm, respectively. For the oxidized form of recombinant Ptc6, the absorption maxima peaks of $\alpha+\beta$, γ (soret), and δ were 528.5 nm, 411.5 nm, and 360.0 nm respectively, and a shoulder peak at 695.0 nm, indicating a His-Fe-Met coordination, was observed (Fig. 1A, inset). The UV/vis absorption spectra of recombinant Ptc6 were similar to the previous results.[4] The redox data were analyzed with a theoretical curve based on the Nernst equation ($n = 1$): $E = E^0 + (RT/nF)\ln([cyt_{oxi}]/[cyt_{red}])$. In good agreement with a previously published report,[4] the redox potential of this protein was +349 mV (Fig. 1B).

Denaturation of oxidized Ptc6 and oxidized *Porphyra yezoensis* cyt c_6 with guanidine hydrochloride (Gdn-

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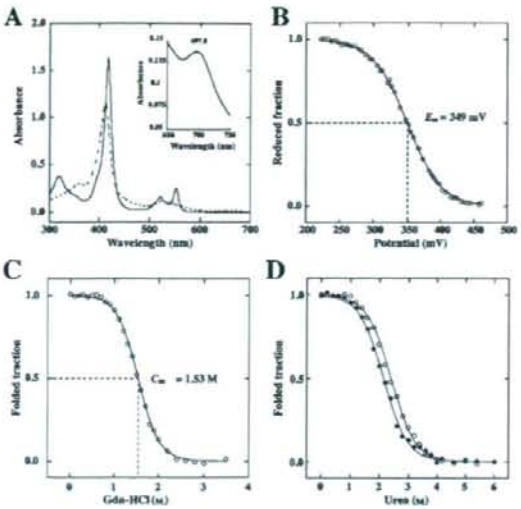

Fig. 1. Physicochemical Properties of Diatom Ptc6.

A, UV/visible spectra of reduced (solid line) and oxidized (broken line) forms of Ptc6. Absorption spectra of 10 μM Ptc6 were measured in 10 mM sodium phosphate (pH 7.0) at 25 °C. The inset shows the 700 nm band of the oxidant form at 80 μM. Sodium dithionite and potassium ferricyanide were used as oxidant and reductant respectively. B, Redox titration of Ptc6. The redox state of the protein was determined by changing the α-band absorption spectrum using a Hitachi U-3310 spectrophotometer. The smooth curve was drawn by the Nernst equation giving the best fit to the data using a Sigma Plot program. C, Gdn-HCl denaturation curves of oxidized Ptc6. Ptc6 was denatured by titration with Gdn-HCl in 10 mM sodium phosphate (pH 7.0) at a protein concentration of 5 μM. D, Urea denaturation curves of oxidized Ptc6 and red algal cyts c_6. Ptc6 and red algal cyts c_6 were denatured by titration with urea in 10 mM sodium phosphate (pH 7.0) at a protein concentration of 5 μM. Ptc6 (circles); red algal cyt c_6 (closed circles).

HCl) and urea as denaturants was measured following previous reports.[7,8] Purification of the red alga *P. yezoensis* cyt c_6 was performed as previously described.[7] The denaturation data were analyzed with a theoretical curve based on $\Delta G_{unf} = -RT \ln K_D = \Delta G^0{}_{unf} - m$[denaturant] by assuming the two-state folding-unfolding transition with the equilibrium constant K_D. The thermodynamic parameter, $\Delta G^0{}_{unf}$, shows the free energy change from the folded state to the unfolded state in the absence of a denaturant, and m shows the dependence of the free energy change ($\Delta G^0{}_{unf}$) on the denaturant concentration. In the case of Gdn-HCl as denaturant, the C_m, $\Delta G^0{}_{unf}$, and m values of the oxidized Ptc6 were 1.53 M, 3.83 kcal/mol, and 2.50 kcal/mol/M respectively (Fig. 1C). The values for Ptc6 were higher than those for the red alga *P. yezoensis* cyt c_6 ($C_m = 0.95$ M, $\Delta G^0{}_{unf} = 2.43$ kcal/mol, $m = 2.56$ kcal/mol/M).[7] In the case of urea, the values for oxidized Ptc6 were 2.39 M, 3.73 kcal/mol, and 1.56 kcal/mol/M respectively. The values for Ptc6 were also higher than those for the red alga *P. yezoensis* cyt c_6 ($C_m = 2.22$ M, $\Delta G^0{}_{unf} = 2.87$ kcal/mol, $m = 1.29$ kcal/mol/M) (Fig. 1D). These results indicate that the structural stability of Ptc6 is higher than that of red algal cyt c_6. The soret peaks for Ptc6 were blue-shifted by urea (from 411.5 nm to 404.5 nm) and Gdn-HCl (from 411.5 nm to 406.5 nm) denaturation. These blue-shifts of the soret peak are indicative of conversion of the heme toward a high-spin form with disruption of heme six coordinated.[9] The soret peaks of the unfolded state of Ptc6 were red-shifted by dialysis against 10 mM sodium phosphate buffer (pH 7.0), and the soret peaks of refolded state of Ptc6 agreed with those of native Ptc6. Thus these results suggest that urea and Gdn-HCl denaturation of Ptc6 are reversible.

Table 1. Crystal Parameters, Data Collection, and Structure Refinement

Data-collection statistics	
Temperature (K)	100
Resolution range (Å)	50.0–1.50
Space group	*I*23
Unit cell parameters (Å)	$a = b = c = 80.380$
Reflections (Measured/Unique)	273393/13706
Completeness (Overall/Outer Shell, %)	97.8/100
R_{merge} (Overall/Outer Shell, %)	4.1/6.4
Redundancy (Overall/Outer Shell)	20.0
Mean [I/s (I)]	32.7
Refinement statistics	
Resolution range (Å)	20.0–1.50
cut-off/reflections used	0.0/13675
R factor/R-free (%)	16.4/20.2%
R.m.s.d. bond length/bond angle (°)	0.009/1.148
Residues in most favourable region (%)	86.1%
Residues in additional allowed region (%)	12.5%
Residues of disallowed region (%)	1.5%

$R_{merge} = \sum |I_i - \langle I \rangle| / \sum I_i$, where I_i is the intensity of an observation and $\langle I \rangle$ is the mean value for its unique reflection; summations are overall reflections. R factor $= \sum_h |F_o(h) - F_c(h)| / \sum hF_o(h)$, where F_o and F_c are the observed and calculated structure-factor amplitudes respectively. The free R factor was calculated with 5% of the data excluded from the refinement. Values in parentheses are for the outer shell, with a resolution within 50.0–1.50 Å.

For crystallization of Ptc6, the purified recombinant protein was dissolved in super-pure water to prepare a concentrated protein solution of 50 mg ml^{-1}. Ptc6 was crystallized by vapor diffusion; the hanging drops used contained a 1:1 mixture of protein and reservoir solution. Cyt c_6 was allowed to crystallize over a reservoir containing 0.1 M Tris–HCl pH 8.5, 0.2 M $MgSO_4$, and 35% Polyethylene Glycol 4000. X-ray diffraction data were collected on the BL-5A (Photon Factory, Tsukuba, Japan). The data set was processed with HKL2000 and scaled with SCALE-PACK.[10] The structure of Ptc6 was determined by molecular replacement using the program MOLREP[11] and the structure of *P. yezoensis* cyt c_6.[12] The structure of Ptc6 was refined with Refmac in the CCP4 program suite. Water molecules were added using a water pick script of CNS, and refinement was continued using REFMAC5.[11] The final model obtained had an R-factor of 16.4% and a free R-factor of 20.2%. Manual model building was carried out using Coot.[13] Solvent molecules were placed at positions where spherical electron density peaks were found above 1.5σ in the $|2F_o - F_c|$ map and above 3.0σ in the $|F_o - F_c|$ map, and where stereochemically reasonable hydrogen bonds were allowed. A summary of data collection and refinement statistics is given in Table 1. The refined crystallographic coordinates and structure factor amplitudes have been deposited in the Protein Data Bank (PDB entry 3dmi).

The first crystal structure of diatom cyt c_6 was determined at 1.5 Å resolution. The crystal belonged to space group I_{23}, with unit cell parameters $a = b = c = 80.38$ Å and one molecule per asymmetric unit. The overall structure of Ptc6 followed the topology of class I c-type cyts (Fig. 2B). An amino acid sequence comparison of diatom Ptc6 with *Chlamydomonas reinhardtii*,