Available online at www.sciencedirect.com
SCIENCE DIRECT®
Carbohydrate Research 338 (2003) 1477–1490
CARBOHYDRATE RESEARCH
www.elsevier.com/locate/carres

β-Glycosylamidine as a ligand for affinity chromatography tailored to the glycon substrate specificity of β-glycosidases

Kazuko Inoue,[a] Jun Hiratake,[a,*] Masaharu Mizutani,[a] Masayasu Takada,[b] Mikio Yamamoto,[b] Kanzo Sakata[a]

[a] *Institute for Chemical Research, Kyoto University, Uji, Kyoto 611-0011, Japan*
[b] *Research Institute, Nihon Shokuhin Kako Co., Ltd., 30 Tajima, Fuji, Shizuoka 417-8530, Japan*

Received 31 January 2003; accepted 16 April 2003

Abstract

An affinity adsorbent for β-glycosidases has been prepared by using β-glycosylamidine as a ligand. β-Glucosylamidine and β-galactosylamidine, highly potent and selective inhibitors of β-glucosidases and β-galactosidases, respectively, were immobilized by a novel one-pot procedure involving the addition of a β-glycosylamine and 2-iminothiolane·HCl simultaneously to a matrix modified with maleimido groups via an appropriate spacer to give an affinity adsorbent for β-glucosidases and β-galactosidases, respectively. This one-pot procedure enables various β-glycosylamidine ligands to be formed and immobilized conveniently according to the glycon substrate specificities of the enzymes. A crude enzyme extract from tea leaves (*Camellia sinensis*) and a β-galactosidase from *Penicillium multicolor* were chromatographed directly on each affinity adsorbent to give a β-glucosidase and a β-galactosidase to apparent homogeneity in one step by eluting the column with glucose or by a gradient NaCl elution, respectively. The β-glucosidase and β-galactosidase were inhibited competitively by a soluble form of the corresponding β-glycosylamidine ligand with an inhibition constant (K_i) of 2.1 and 0.80 μM, respectively. Neither enzyme was bound to the adsorbent with a mismatched ligand, indicating that the binding of the glycosidases was of specific nature that corresponds to the glycon substrate specificity of the enzymes. The ease of preparation and the selective nature of the affinity adsorbent should promise a large-scale preparation of the affinity adsorbent for the purification and removal of specific glycosidases according to their glycon substrate specificities.

Keywords: β-Glycosylamidine; Affinity ligand; Affinity chromatography; Glycon substrate specificity; Tea leaf β-glucosidase; *Penicillium multicolor* β-galactosidase

1. Introduction

Glycosidases are widespread in nature and serve a number of important biological functions such as intestinal digestion, post-translational modification of glycoproteins and the catabolism of glycoconjugates in lysosome.[1,2] One of the striking features of glycosidases is their diversity: the glycoside hydrolases are classified into 90 families according to their amino acid sequences (URL: http://afmb.cnrs-mrs.fr/CAZY/GH.html),[3] and each family contains a number of glycosidases with different glycon substrate specificities. The sequence-based classification of glycoside hydrolases is highly useful in characterizing the enzymes from the structural point of view,[4] but the substrate specificity with respect to the glycon moiety still serves a primary or, in some cases, the only lead in isolating and characterizing unknown or structurally undefined glycosidases. It is therefore highly desirable to develop an efficient method for separating glycosidases according to their glycon substrate specificities, when in particular no other chemoenzymatic information is available. Affinity chromatography[5,6] has often been a method of choice for

Abbreviations: *p*NP-β-Glc, *p*-nitrophenyl β-D-glucopyranoside; *o*NP-β-Gal, *o*-nitrophenyl β-D-galactopyranoside; *p*NP-β-Xyl, *p*-nitrophenyl β-D-xylopyranoside; GMBS, *N*-(γ-maleimidobutyryloxy)succinimide ester; SDS-PAGE, sodium dodecylsulfate polyacrylamide gel electrophoresis.

* Corresponding author. Tel.: +81-774-383231; fax: +81-774-383229.
E-mail address: hiratake@scl.kyoto-u.ac.jp (J. Hiratake).

0008-6215/03/$ - see front matter © 2003 Elsevier Science Ltd. All rights reserved.
doi:10.1016/S0008-6215(03)00201-5

this purpose. So far a number of glycosides[7–10] and their analogs such as thioglycosides,[11–14] glycamines,[15] glycosylamides[16–20] have been used successfully as ligands to achieve specific binding of glycosidases. The use of glycosidase inhibitors such as deoxynojirimycin and its analogs[21–27] was also found effective for higher affinity and selectivity, but is difficult in practice due to limited access to a set of inhibitors according to the glycon substrate specificities of glycosidases. We previously reported that β-glycosylamidines were highly potent and selective inhibitors of β-glycosidases.[28] The inhibition was selective according to the enzyme's glycon- and stereospecificities, and a series of β-glycosylamidines with a different glycon moiety was easily synthesized[28] from the corresponding sugar via the β-glycosylamines.[29] Furthermore, the β-glycosylamidines are chemically stable under weakly acidic conditions where most glycosidases are stable and exhibit the maximum activities.[28] We reasoned therefore that the β-glycosylamidines would serve as effective ligands for the affinity chromatography of β-glycosidases, ligands tailored to the glycon substrate specificities of the enzymes.

In this paper, we report the preparation and characterization of the affinity adsorbents with β-glycosylamidine as ligands. In preparing the adsorbent, a novel one-pot procedure for in situ formation and immobilization of β-glycosylamidines has been developed so as to tailor the ligand conveniently to the glycon substrate specificity of glycosidases. The affinity adsorbents with β-glucosyl- and β-galactosylamidines as ligands were used successfully for one-step purification of a β-glucosidase from tea leaves and a β-galactosidase from a mold, respectively. The selective nature of the ligand–enzyme interaction is shown by chromatographing the enzymes on the adsorbent with a 'mismatched' ligand.

2. Results

2.1. Synthesis of β-glucosyl- and β-galactosylamidines 2a and 2b as soluble ligands

Before immobilizing each of the β-glycosylamidines as an affinity ligand, a soluble form of the ligand was synthesized to examine the immobilization method as well as the inhibitory activity. Since β-glycosylamidines are synthesized readily from the corresponding β-glycosylamines and thioimidates without protecting the hydroxy groups,[28] a one-pot procedure for in situ formation and trapping of the β-glycosylamidine was developed by using a cyclic thioimidate and a maleimide (Scheme 1).

Thus, β-glucosylamine (**1a**) and β-galactosylamine (**1b**) were treated with 2-iminothiolane·HCl in the presence of *N*-benzylmaleimide. The β-glucosyl- and β-galactosylamidines formed in situ from **1a** and **1b** with the cyclic thioimidate, respectively, were trapped immediately by the maleimido group to give the corresponding β-glycosylamidines **2a** and **2b**. The reaction was clean and gave the amidines **2a** and **2b** as the sole product (^{13}C NMR). The formation of the intermediate β-glucosylamidine with a terminal SH was confirmed by ^{1}H NMR when the amine **1a** was treated with 2-iminothiolane·HCl alone, and the subsequent addition of the maleimide gave **2a**. However, the best yield was obtained when all the reagents were mixed together at once to ensure facile trapping of the unstable thiol. This one-pot procedure was used successfully for immobilizing the β-glycosylamidine as a ligand onto a matrix with maleimido groups at the terminal via an appropriate tether (vide infra).

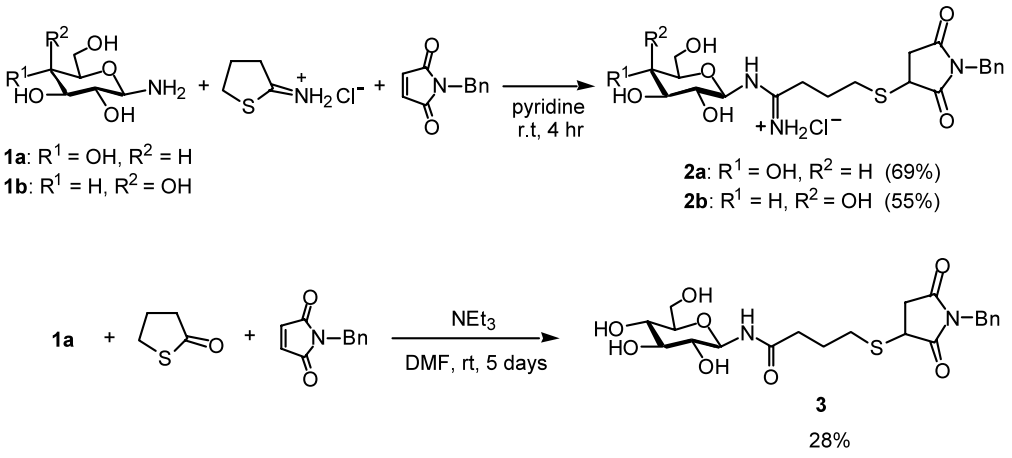
The structurally analogous, but neutral β-glucosylamide (**3**) was also synthesized as a control compound for the inhibition experiment. The same one-pot procedure using γ-thiobutyrolactone in place of 2-iminothiolane·HCl was used for the synthesis, but the reaction was rather slow and gave the β-glucosylamide (**3**) in 28% yield after 5 days.

2.2. Inhibition

The β-glycosylamidines **2a**, **b** and the amide **3** were tested for their inhibitory activities against several glycosidases with different glycon specificities (Table 1).

The β-glucosylamidine (**2a**) was an extremely potent inhibitor of the β-glucosidases from *Aspergillus niger* and *Trichoderma viride* with an inhibition constant (K_i) of 0.008 and 0.25 μM, respectively. The inhibition was competitive with respect to the substrate [*p*-nitrophenyl β-D-glucopyranoside (*p*NP-β-Glc)]. No slow-binding kinetics[30] was observed in spite of the strong inhibition.[31,32] Interestingly, a β-glucosidase from almond was not inhibited at all by **2a** ($IC_{50} = 14$ mM), although a related β-glucosylamidine inhibited the enzyme with a K_i of 73 μM.[28]

The β-galactosylamidine (**2b**) was also a potent inhibitor of the β-galactosidases with K_i values in the μM range. In addition, a high degree of selectivity with respect to the glycon moiety was observed: the β-galactosylamidine (**2b**) inhibited the β-glucosidases much less potently than **2a** and did not inhibit the β-xylosidase at all. The β-glucosylamide (**3**), a neutral analog of **2a**, was much less inhibitory than the amidine **2a**, but was still more inhibitory than β-galactosylamidine (**2b**) towards the β-glucosidases. The β-glycosylamidines **2a** and **2b** were thus found to bind tightly and selectively to the β-glycosidases with the corresponding glycon substrate specificity, and were used as an affinity ligand for the purification of glycosidases according to the glycon substrate specificities.



Scheme 1. Synthesis of soluble ligands.

Table 1
Inhibitory activities of β-glycosylamidines **2a** and **2b** and β-glucosylamide **3**

Glycosidases	Origin	K_i (μM)		
		2a	**2b**	**3**
β-Glucosidase	*A. niger* [a]	0.0080	410	4.9
	T. viride [b]	0.25	660	19
	Almond	14,000 [c]	M[d]	M
β-Galactosidase	*A. oryzae*	M	16	M
	E. coli	M	6.0	M
β-Xylosidase	*A. pulverulentus*	M	NI [e]	M

[a] *Aspergillus niger*.
[b] *Trichoderma viride*.
[c] IC_{50}.
[d] Not determined.
[e] No inhibition at 2.5 mM inhibitor.

2.3. Preparation of affinity adsorbent with β-glycosylamidine as ligand

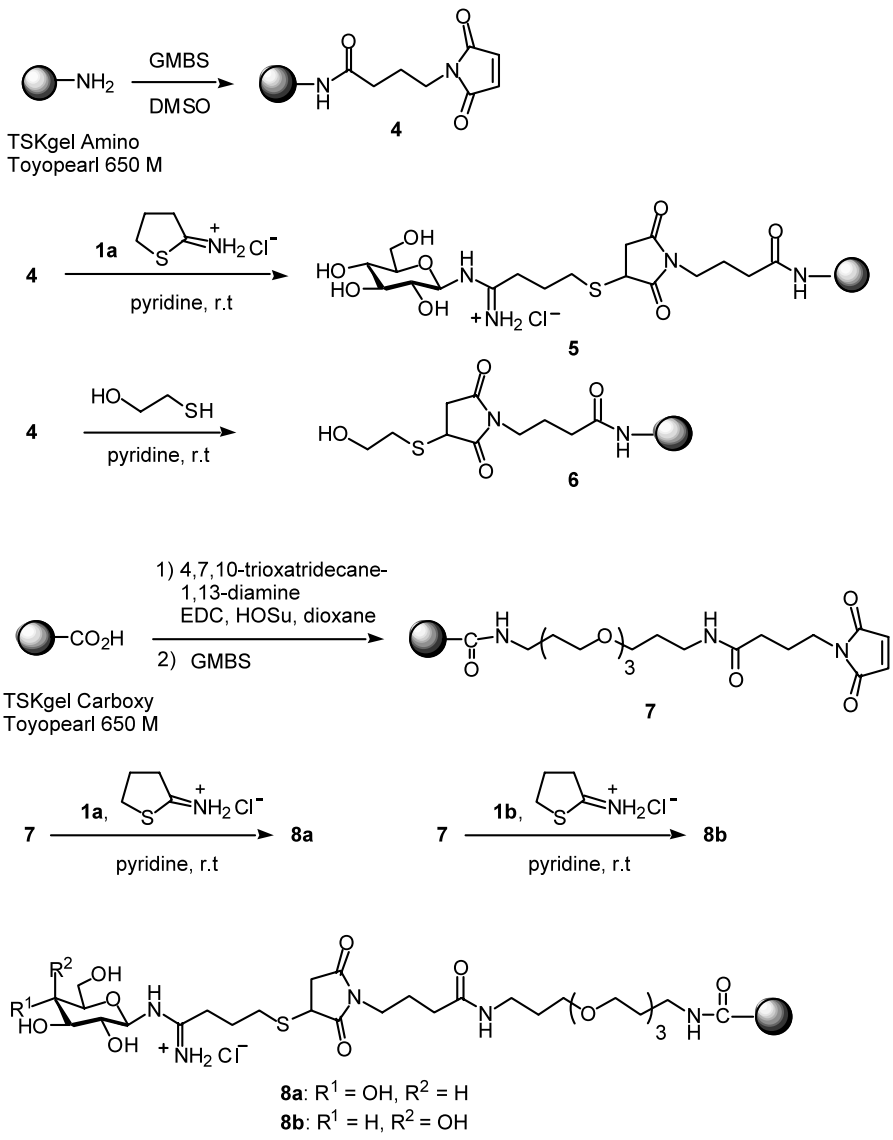
The one-pot procedure described above involving the in situ formation and trapping of the β-glycosylamidines was used successfully for the immobilization of the ligand onto the matrix (Scheme 2). A solid support with amino groups (TSKgel AF-Amino Toyopearl 650M®) was treated with a heterobifunctional cross-linking reagent, *N*-(γ-maleimidobutyryloxy)succinimide ester (GMBS) to introduce maleimido groups via a spacer of four carbon atoms in length. The resulting gel **4** was suspended in dry pyridine and was treated with an excess amount of β-glucosylamine (**1a**) and 2-iminothiolane·HCl simultaneously to immobilize the β-glucosylamidine as a ligand to yield the affinity adsorbent **5**. The amount of the ligand was calculated as 86 μmol/mL of the gel from the moles of the amino groups of the matrix before and after the modification with GMBS. This value was corresponding to 98% moles of all the titrable amino groups of the matrix (88 μmol/mL gel). A control gel **6** without the ligand was prepared by treating the gel **4** with 2-mercaptoethanol under the same reaction conditions.

Among several factors affecting the adsorptive characteristics of affinity gel,[33] a spacer arm between the ligand and the solid support is highly important in reducing the unfavorable steric interaction between the proteins and the matrix.[33,34] Hence we prepared the affinity adsorbent **8a** with β-glucosylamidine as a ligand via a longer spacer by adding **1a** and 2-iminothiolane·HCl to a matrix **7** with maleimido groups via a spacer arm of 19 atoms in length. The same matrix **7** was used for the preparation of the affinity adsorbent **8b** with β-galactosylamidine as a ligand by adding β-galactosylamine (**1b**) in place of **1a** at the last stage of the preparation.

Since the glycosylamidines are less stable in alkaline than in acidic to neutral solutions,[28] the affinity adsorbents **5**, **8a** and **8b** were washed with 0.1 M citrate buffer (pH 6) and were suspended and stored in the same buffer.

2.4. Affinity chromatography of tea leaf β-glucosidase

The adsorptive characteristics of the affinity adsorbent **5** were examined by using a β-glucosidase from tea leaves. Tea leaves (*Camellia sinensis* var. *sinensis* cv. Yabukita) were reported to contain at least three β-glucosidases,[35] but none of them has been isolated. A crude enzyme extracted from the acetone powder of tea leaves[35] was applied first to a column (0.8 × 4 cm) charged with the control gel **6**. A high β-glucosidase activity was observed in the fractions that passed through the column without adsorption. The fractions containing the β-glucosidase activity were collected and applied to the second column charged with the affinity adsorbent **5** with β-glucosylamidine as a ligand. The column was washed with 0.1 M citrate buffer (pH 6) and 1 M NaCl successively, and



Scheme 2. Preparation of affinity adsorbents.

was eluted with 0.5 M glucose to give a distinct β-glucosidase activity in the glucose eluent (Fig. 1).

The sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) of each fraction under reducing conditions is shown in Fig. 2. The pass-though fraction from the control gel **6** (lane 2) contained as many protein bands as the crude enzyme extract (lane 1), but one protein band was missing in the fraction that passed through the affinity gel **5** (lane 3). The missing protein was not eluted by washing the column with 1 M NaCl (lane 4), but was recovered as an apparently single band by elution with 0.5 M glucose (lane 5). The protein eluted by glucose was a β-glucosidase with an approximate molecular mass of 65 kDa.

The specific activity of the β-glucosidase was calculated as 2.1×10^{-2} unit/mg protein, which was 20-fold higher than that of the crude enzyme extract. A total of 8% of the β-glucosidase activity was recovered (Table 2).

The capacity of the affinity adsorbent **5** was determined by applying an excess amount of the crude enzyme (0.20 unit of β-glucosidase per mL of the adsorbent). The column was washed with 0.1 M citrate buffer (pH 6) and 1 M NaCl, successively, and was eluted with 0.5 M glucose by the same way. The chromatogram and the SDS-PAGE of the eluates are shown in Fig. 3a and b, respectively.

The β-glucosidase was bound selectively (fraction 1, lane 2), but the unbound β-glucosidase was found in the pass-through fractions (fractions 2–5, lane 3), because a large excess amount of the enzyme was applied to the column. The bound enzyme, however, was eluted specifically with glucose to give the highly purified β-glucosidase (fraction 13, lane 6). The capacity of the column was calculated from the total amount of the β-glucosidase eluted from the column and was found to be 0.022 unit of β-glucosidase per mL of the affinity gel.

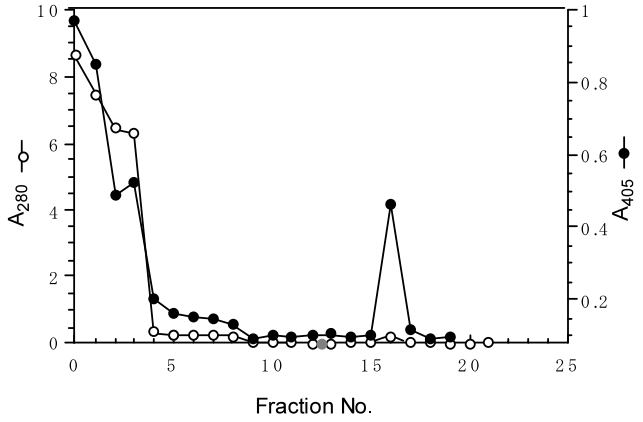


Fig. 1. Affinity chromatography of tea leaf β-glucosidase on the affinity adsorbent **5**. Protein content (○, absorbance at 280 nm), β-glucosidase activity (●, absorbance at 405 nm) with *p*NP-β-Glc as substrate. Fr. 0, crude enzyme; Fr. 1, pass-through fraction from the control gel **6**; Fr. 2, pass-through fraction from the adsorbent **5**; Fr. 3–10, washing with 0.1 M citrate buffer (pH 6); Fr. 11–15, washing with 1 M NaCl; Fr. 16–21, elution with 0.5 M glucose; Fr. 22–25, washing with 5 M urea.

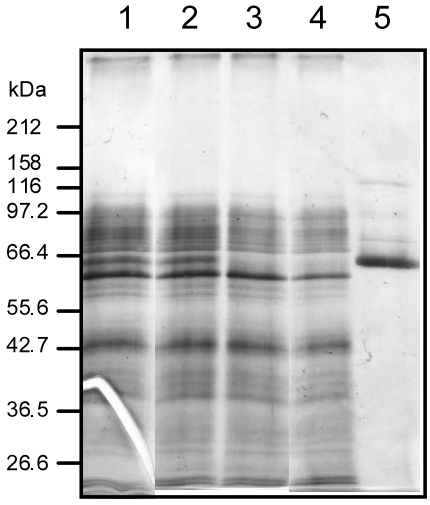


Fig. 2. SDS-PAGE of tea leaf β-glucosidase eluted from the affinity adsorbent **5**. The proteins were separated on 10% (w/v) SDS-PAGE and were visualized by silver staining. Lane 1, crude enzyme; lane 2, pass-through fraction from the control gel **6**; lane 3, pass-through fraction from the affinity adsorbent **5**; lane 4, washing with 0.1 M citrate buffer (pH 6.0); lane 5, eluate with 0.5 M glucose.

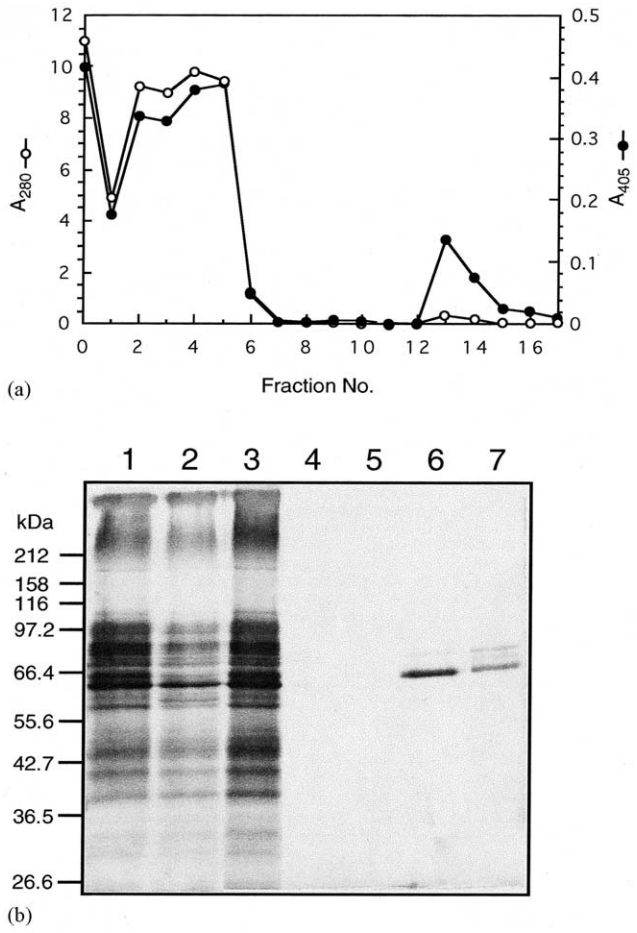


Fig. 3. (a) Chromatography of excess tea leaf β-glucosidase on the affinity adsorbent **5**. A total of 0.2 units of β-glucosidase per mL of the adsorbent was applied to the column. Protein content (○, absorbance at 280 nm), β-glucosidase activity (●, absorbance at 405 nm) with *p*NP-β-Glc as substrate. Fr. 0, crude enzyme; Fr. 1–5, pass-through fractions; Fr. 6–7, washing with 0.1 M citrate buffer (pH 6); Fr. 8–12, washing with 1 M NaCl; Fr. 13–15, elution with 0.5 M glucose; Fr. 16–17, elution with 1 M glucose; (b) SDS-PAGE of the eluates. The proteins were separated on 10% (w/v) SDS-PAGE and were visualized by silver staining. Lane 1, crude enzyme; lane 2, pass-through fraction (fraction No. 1); lane 3, pass-through fraction (fraction No. 4); lane 4, washing with 0.1 M citrate buffer (pH 6.0) (fraction No. 7); lane 5, washing with 1 M NaCl (fraction No. 9); lane 6, eluate with 0.5 M glucose (fraction No. 13); lane 7, eluate with 1 M glucose (fraction No. 16).

Table 2
Purification of β-glucosidase from tea leaves with affinity adsorbent **5**

Enzyme	Total protein (μg)	Total activity [a] (unit)	Specific activity (unit/mg)	Purification (-fold)	Yield (%)
Crude	3200	3.3×10^{-3}	1.0×10^{-3}	1	100
Affinity purified [b]	13	2.7×10^{-4}	2.1×10^{-2}	20	8.2

[a] Activity was measured with *p*NP-β-Glc as substrate (see Section 4).
[b] Fraction 16 in Fig. 1.

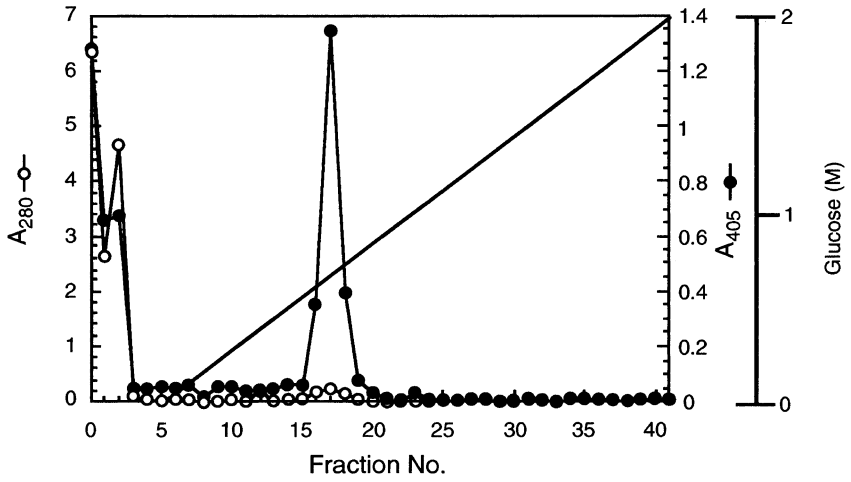


Fig. 4. Elution of tea leaf β-glucosidase from the affinity adsorbent **5** by glucose gradient. Protein content (○, absorbance at 280 nm), β-glucosidase activity (●, absorbance at 405 nm) with *p*NP-β-Glc as substrate. Fr. 0, pass-through fraction; Fr. 1–5, washing with 0.1 M citrate buffer (pH 6); Fr. 6–40, glucose gradient (0–2 M).

Since the β-glucosidase was eluted with 0.5 M glucose, a linear gradient of glucose was used for elution (Fig. 4).

The crude enzyme was applied to the adsorbent **5**, and the column was washed with 0.1 M citrate buffer (pH 6). The bound β-glucosidase was eluted by increasing the concentration of glucose linearly from 0 to 2 M. A single peak of β-glucosidase activity was eluted at 0.34 M of glucose, and no other peaks of β-glucosidase activity were observed in the fractions eluted with higher glucose concentrations. The β-glucosidase eluted with 0.34 M glucose gave a single band on SDS-PAGE and was identical to that obtained in Figs. 2 and 3.

The crude β-glucosidase from tea leaves was also chromatographed on the affinity adsorbent **8a** with a long spacer (Fig. 5).

The basic adsorptive characteristics of the ligand were not affected significantly by the length of the spacer, giving the purified β-glucosidase with 17-fold higher specific activity than that of the crude enzyme. The SDS-PAGE also confirmed the purity of the eluted enzyme (data not shown).

Finally, the affinity purified β-glucosidase from tea leaves was characterized by using *p*NP-β-Glc as substrate. The optimum pH for the enzyme activity was 4.5,

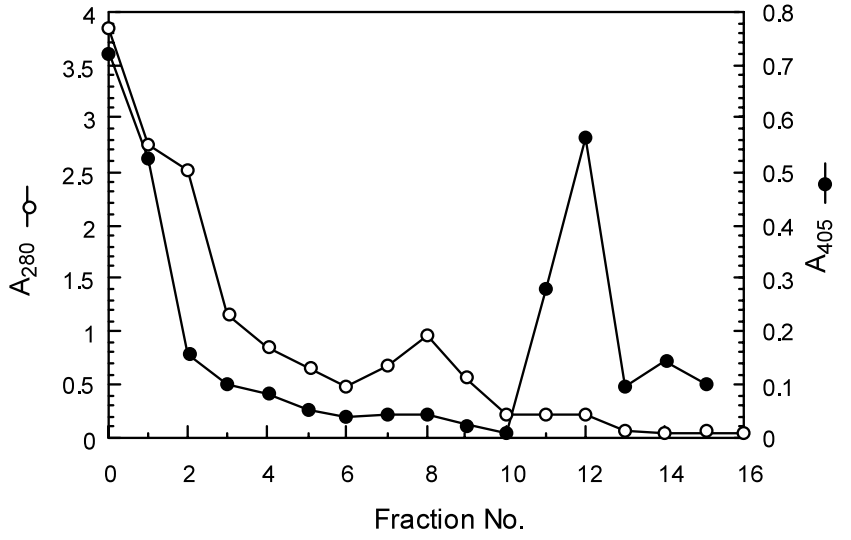


Fig. 5. Affinity chromatography of tea leaf β-glucosidase with the affinity adsorbent **8a**. Protein content (○, absorbance at 280 nm), β-glucosidase activity (●, absorbance at 405 nm) with *p*NP-β-Glc as substrate. Fr. 0, crude enzyme; Fr. 1, pass-through fraction; Fr. 2–5, washing with 0.1 M citrate buffer (pH 6); Fr. 6–10, washing with 1 M NaCl; Fr. 11–15, elution with 1 M glucose; Fr. 16, washing with 5 M urea.

and the kinetic parameters were determined as K_m = 0.70 mM, V_{max} = 1.6 μmol/min/mg protein at this pH at 37 °C. Under the same conditions as used for the chromatography, the β-glucosidase was inhibited competitively by the soluble ligand **2a** with a K_i of 2.1 μM.

2.5. Affinity chromatography of β-galactosidase from *Penicillium multicolor*

The affinity adsorbent **8b** with β-galactosylamidine as a ligand was characterized by using a β-galactosidase from *P. multicolor*. A preliminary experiment revealed that the β-galactosidase was bound to the adsorbent **8b**, but was eluted from the column with 0.1 M NaCl, as well as with 1 M galactose. Therefore, the column was eluted with a linear gradient of NaCl from 0 to 250 mM after the β-galactosidase had been adsorbed. The elution profile and the SDS-PAGE of the eluates are shown in Fig. 6a and b, respectively.

A major β-galactosidase activity was eluted at 120–140 mM NaCl as a single peak. The SDS-PAGE of the fractions showed that the β-galactosidase of an approximate molecular mass of 130 kDa was purified to almost homogeneity. The specific activity of the β-galactosidase was calculated as 173 unit/mg protein, which was 3.2-fold higher than that of the crude enzyme (Table 3). The recovery of the β-galactosidase activity was 93%.

The K_m of the purified β-galactosidase for *o*NP-Gal was determined as 0.46 mM at pH 5.0. The inhibition of the β-galactosidase by the soluble ligand **2b** was also examined under the same conditions as used for the affinity chromatography (20 mM acetate buffer, pH 5.0). The enzyme was inhibited competitively by the β-galactosylamidine (**2b**) with a K_i of 0.80 μM.

2.6. Affinity chromatography with 'mismatched' ligand

The β-glucosidase from tea leaves and the β-galactosidase from *P. multicolor* were thus bound to the affinity

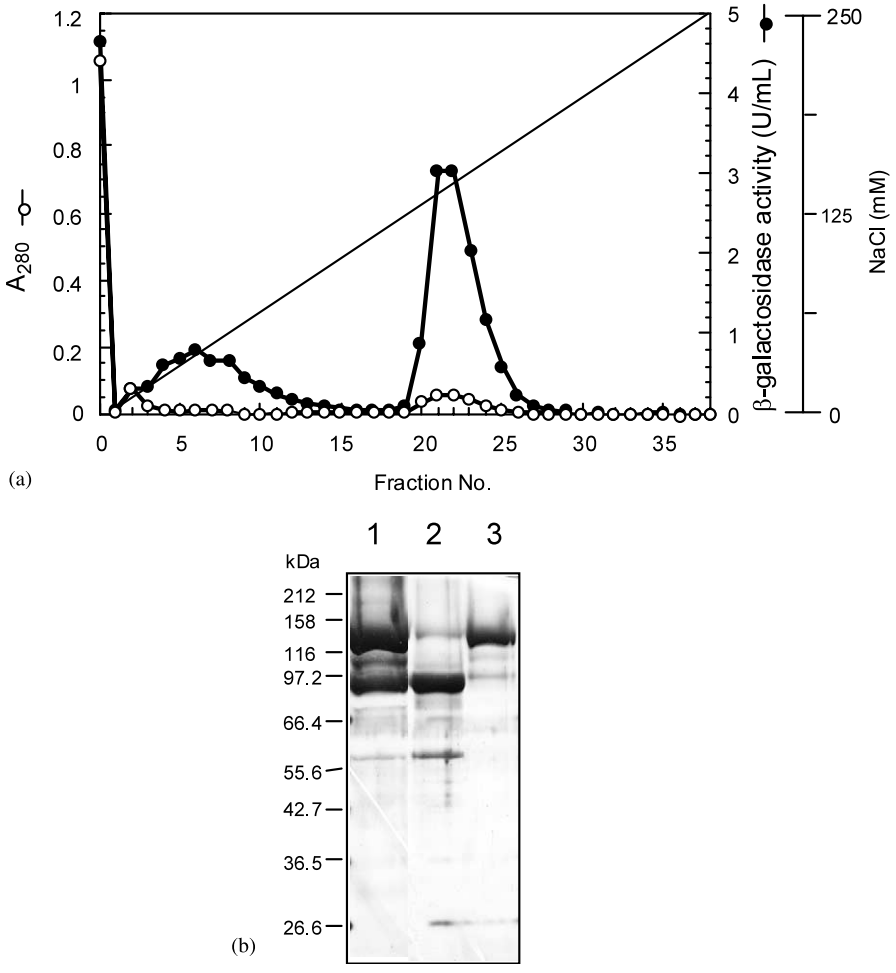


Fig. 6. (a) Affinity chromatography of β-galactosidase from *P. multicolor* with β-galactosylamidine affinity adsorbent **8b**. Protein content (○, absorbance at 280 nm), β-galactosidase activity (●, U/mL) with *o*NP-β-Gal as substrate. Fr. 0, crude enzyme; Fr. 1–38, elution with 0–250 mM NaCl gradient; (b) SDS-PAGE of the eluates. The proteins were separated on 10% (w/v) SDS-PAGE and were visualized by silver staining. Lane 1, crude enzyme; lane 2, fraction No. 3; lane 3, fraction No. 21.

Table 3
Purification of β-galactosidase from *P. multicolor* with affinity adsorbent **8b**

Enzyme	Total protein (μg)	Total activity [a] (unit)	Specific activity (unit/mg)	Purification (-fold)	Yield (%)
Crude	432	23.3	53.9	1	100
Affinity purified [b]	125	21.6	173	3.2	93

[a] Activity was measured with *o*NP-β-Gal as substrate (see Section 4).
[b] Fraction 20–25 in Fig. 6a.

adsorbents **8a** and **8b**, respectively, and were eluted from the adsorbent by the corresponding sugar. It is necessary, however, to see if the binding of the enzymes was due to the specific interaction between the enzyme and the ligand, the interaction according with the glycon substrate specificity of the enzymes. We therefore chromatographed each enzyme on the affinity adsorbent with a 'mismatched' or unrelated ligand. Thus, the crude β-glucosidase from tea leaves and the crude β-galactosidase from *P. multicolor* were chromatographed on the affinity adsorbents **8b** and **8a** with β-galactosylamidine and β-glucosylamidine as the ligand, respectively. The elution profiles are shown in Fig. 7a and b.

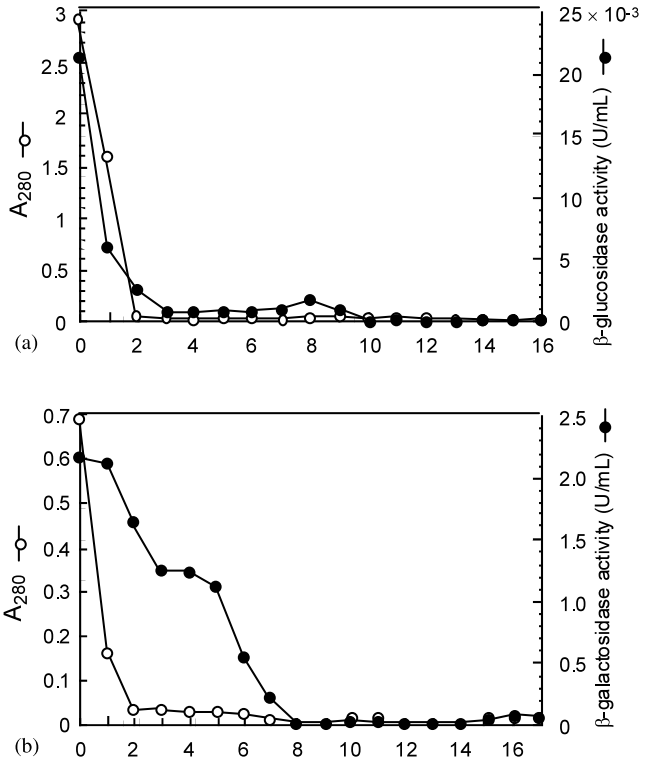


Fig. 7. Chromatography of glycosidases with affinity adsorbent with 'mismatched' ligand: (a) β-glucosidase from tea leaves chromatographed on **8b**. Fr. 0, crude enzyme; Fr. 1–6, washing with 0.1 M citrate buffer (pH 6); Fr. 7–10, washing with 1 M NaCl; Fr. 11–13, elution with 0.5 M glucose; Fr. 14–17, elution with 1 M galactose. (b) β-Galactosidase from *P. multicolor* chromatographed on **8a**. Fr. 0, crude enzyme; Fr. 1–5, washing with 20 mM citrate buffer (pH 5); Fr. 6–9, washing with 1 M NaCl; Fr. 10–14, elution with 1 M galactose; Fr. 15–17, elution with 1 M glucose.

As expected, all the β-glucosidase activity passed through the adsorbent **8b** without adsorption. No β-glucosidase activity was recovered by elution with 0.5 M glucose (fractions 11–13) or with 1 M galactose (fractions 14–17). The β-galactosidase, on the other hand, was slightly bound to the adsorbent **8a**, but was eluted almost completely by washing with a low concentration of a buffer (fractions 1–5). No β-galactosidase activity was recovered by elution with either 1 M galactose (fractions 10–14) or with 1 M glucose (fractions 15–17).

3. Discussion

The present paper describes: (1) the preparation of the affinity adsorbents with β-glycosylamidines as ligands by a novel one-pot procedure involving in situ formation and immobilization of the β-glycosylamidine ligands onto a matrix; (2) the affinity as measured by inhibitory activity of the soluble form of the ligand towards several β-glycosidases; and (3) the evaluation of the affinity adsorbents by direct application of a crude β-glucosidase extract from tea leaves and a crude β-galactosidase from *P. multicolor* as representative enzymes for affinity chromatography. The use of a commercially available cyclic thioimidate, 2-iminothiolane·HCl, combined with *N*-benzylmaleimide has enabled the soluble form of the β-glycosylamidine ligands **2a** and **2b** to be prepared in one step in fairly good yields from the corresponding β-glycosylamines **1a** and **1b**, respectively. This one-pot procedure was applied successfully to the preparation of the affinity adsorbents **5**, **8a** and **8b** with β-glucosyl- or β-galactosylamidine as a ligand by using a matrix modified with maleimido groups via an appropriate spacer. The ligand was readily tailored to the glycon substrate specificity of the enzyme by choosing an appropriate β-glycosylamine added at the last stage of the synthesis (Scheme 2). Neither the isolation of the intermediate β-glycosylamidine nor the protection of the hydroxy and the amidino groups was necessary for the immobilization. Since the β-glycosylamidine was formed directly from the corresponding β-glycosylamine, this method is applicable, in principle, to any glycosylamidine ligand including those derived from

oligosaccharides, provided the precursor glycosylamines are available.[36] We also prepared an affinity adsorbent for β-*N*-acetylhexosaminidases by using a β-glycosylamine derived from *N*-acetyl-D-glucosamine[36] by the same procedure (unpublished result).

The β-glycosylamidines **2a** and **2b** represented a soluble form of the ligand tested for their affinity for the glycosidases with varying glycon substrate specificities. The results shown in Table 1 have revealed that the β-glycosylamidines **2a** and **2b** served as highly potent and selective inhibitors of the β-glycosidases. The degree of inhibition was dependent mostly on the structure of the glycon moiety of the amidines. A β-glucosidase from *A. niger*, for example, was strongly inhibited by **2a** with a K_i of 8 nM, but not by **2b** ($K_i = 410$ μM). Although the positive charge of the glycosylamidines was essential for strong inhibition,[28] the β-glucosylamide (**3**) was still 30–80 times more inhibitory towards β-glucosidases than the β-galactosylamidine **2b**. These results, along with the absence of a slow-binding kinetics in their inhibition,[31] suggested that the β-glycosylamidines served as a substrate analog, where the glycon moiety was primarily recognized like substrates by the glycosidases. These characteristics are suitable for an affinity ligand for separating the glycosidases according to their glycon substrate specificities. Almond β-glucosidase, however, was not inhibited at all by **2a**, although this enzyme was inhibited with a K_i of 73 μM by a β-glucosylamidine with a benzyl group attached directly to the imino carbon.[28] Since aryl glucosides are particularly good substrates for this enzyme,[37] the structure of the 'aglycon' moiety in **2a** probably did not match the aglycon substrate specificity of this enzyme. A number of plant β-glucosidases, including the β-glucosidase from almond, belong to the family 1 glycoside hydrolases[38,39] and exhibit a high degree of specificity for the aglycon moiety.[40] Since the inhibition potency was highly influenced by the structure of the aglycon moiety, the affinity and the selectivity of β-glycosylamidines as affinity ligand could be improved by incorporating an appropriate aglycon according to the aglycon substrate specificity of the enzyme.

The affinity adsorbent **5** with β-glucosylamidine as a ligand was highly useful in isolating a β-glucosidase from tea leaves, effecting one-step purification of an enzyme with a molecular mass of 65 kDa from a crude enzyme extract (Figs. 1 and 2). The isolated enzyme was inhibited by **2a** with a K_i of 2.1 μM. The apparent degree of purification and the yield of the enzyme were calculated as 20-fold and 8%, respectively (Table 2), but these values are underestimated by the presence of at least two other β-glucosidases[35] which did not bind to the adsorbent under the conditions used for the experiment. The binding capacity of the adsorbent **5** seems rather lower than that anticipated from the calculated amount of the ligand, but the adsorbent was stable enough to repeat the chromatography for more than 20 times over 2 months without decreasing the performance (data not shown). Interestingly, the β-glucosidase from *A. niger*,[41] which was inhibited rather strongly by **2a** (Table 1), was bound only weakly to the adsorbent **5** and was eluted from the column by washing with a low concentration of NaCl. The adsorptive characteristics of affinity adsorbents are affected by several factors such as the steric interaction between the enzyme and the matrix, the accessible surface area, the particle size and the ability of the solute to diffuse in and out of the microporous environment.[42,33] As far as the tea leaf β-glucosidase is concerned, the unfavorable steric interaction seems unlikely, because the molecular mass of the β-glucosidase was not so large (65 kDa), and the basic adsorptive characteristics of **8a** with a long spacer was almost the same as that of **5**. The nature of the counter anion might affect the adsorptive characteristic of the β-glucosylamidine ligand, because the ionic interaction between the amidinium cation and the glycosidases contributed significantly to the binding energy (Table 1). If a carboxylate group in the enzyme active site is involved in the ionic interaction with the glycosylamidine, then the presence of an exogenous acetate and citrate as a counter anion of the glycosylamidine ligand may disfavor the binding of the enzyme by competing with the carboxylate of the enzyme. The effect of the counter anion on the inhibition is now under investigation.

The affinity adsorbent **8b** with β-galactosylamidine as a ligand was also effective in isolating a β-galactosidase from *P. multicolor* (Fig. 6). In this particular case, the enzyme was eluted by a linear gradient of NaCl, but a β-galactosidase with a molecular mass of 130 kDa was purified to almost homogeneity in one step with a recovery of the enzyme activity of more than 90%. The isolated β-galactosidase was found to bind to the soluble form of the ligand **2b** with more than 500 times higher affinity than that for the substrate.

A question may arise here if the protein may have been purified by ion-exchange effects of the adsorbent, because the ligand is permanently and positively charged at the pH used for the chromatography. This was tested by chromatographing the β-glycosidases on the affinity adsorbent with a mismatched ligand (Fig. 7a and b). The adsorptive characteristics shown here clearly indicated that the enzyme was bound to the ligand with specific interaction between the glycon moiety of the ligand and the enzyme. This agreed well with the results of inhibition with different glycosidases (Table 1), where the glycon moiety of the β-glycosylamidine significantly contributed to the overall affinity to the enzymes.

In conclusion, the β-glycosylamidines were found effective as an affinity ligand for separating β-glycosidases according to the glycon substrate specificity of the enzymes. This absorptive characteristic reflected the fact

that the β-glycosylamidines serve as substrate analogs, but the analogs strongly interacting with the enzyme by the presence of a positive charge near the anomeric carbon to mimic the oxocarbenium ion intermediate.[28] Hence the β-glycosylamidine ligand successfully recruited the binding energy of the enzyme towards the substrate to achieve selective binding to the corresponding glycosidases. This property, along with the ease of synthesis, should promise useful applications not only for purifying a target glycosidase, but also for removing an undesirable contaminating enzyme from the sample, based on the glycon substrate specificities.

4. Experimental

4.1. General methods

Pyridine was distilled from CaH_2 and stored over 5 Å molecular sieves. Dry *N*,*N*-dimethylformamide (DMF) and dimethyl sulfoxide (Me_2SO) were purchased from Wako Pure Chemical Industries. *N*-Benzylmaleimide and 2-iminothiolane·HCl were purchased from Pierce Chemical Co. *N*-(γ-Maleimidobutyryloxy)succinimide ester (GMBS) was synthesized according to the literature.[43] TSKgel AF-Amino Toyopearl 650M and TSK gel AF-Carboxy Toyopearl 650M were purchased from Tosoh Co., Japan. *p*-Nitrophenyl β-D-glucopyranoside (*p*NP-β-Glc), *o*-nitrophenyl β-D-galactopyranoside (*o*NP-β-Gal) and *p*-nitrophenyl β-D-xylopyranoside (*p*NP-β-Xyl) were purchased from Sigma Chemical Co. Other chemicals were obtained from commercial sources and were of the highest purity available. A β-galactosidase from *P. multicolor* was a generous gift from Amano Enzyme Inc., Japan. Reversed-phase medium-pressure column chromatography was performed on a YFLC system (Yamazen Co., Osaka, Japan) equipped with an ULTRA PACK™ octadecyl silica gel column (ODS-S-50B, 50 μm, 120 Å; Yamazen) by monitoring the absorbance at 254 nm or the conductivity of the eluent. ^1H and ^{13}C NMR spectra were recorded on a Varian VXR-200 (200 MHz) spectrometer using tetramethylsilane (for $CDCl_3$) or 3-(trimethylsilyl)propanesulfonic acid sodium salt (for D_2O) as an internal standard. Chemical shifts were recorded in ppm with the internal standard at $\delta_H = 0.00$. Elemental analyses were performed on a Yanaco MT-5. Mass spectra were obtained on a JEOL JMS700 spectrometer. A Shimadzu UV-1600 UV–Vis spectrophotometer was used for enzyme assay. Protein content was measured by Coomassie protein assay reagent kit (Pierce) with bovine serum albumin (BSA) as standard.

4.2. β-Glucosylamidine (2a)

β-Glucosylamine (**1a**)[29] (179 mg, 1.0 mmol) was suspended in dry pyridine (5 mL) at 0 °C. 2-Iminothiolane·HCl (138 mg, 1.0 mmol) and *N*-benzylmaleimide (187 mg, 1.0 mmol) were added to the suspension, and the mixture was stirred for 4.5 h at 0 °C under an argon atmosphere. The mixture gave a clear solution as the reaction proceeded. After the reaction had been completed (^1H NMR), the reaction mixture was evaporated to dryness. The residual oil was dissolved in water and washed with AcOEt to remove hydrophobic impurities. The aqueous layer was filtered through a MILLEX-GV filter (0.22 μm, Millipore). The filtrate was applied to a medium-pressure ODS column (ODS-S-50B) equilibrated with water, and the column was eluted with a linear gradient from water to 10% MeOH at a flow rate of 6 mL/min. The fractions containing the product eluted at 5% MeOH were collected and lyophilized to afford the β-glucosylamidine (**2a**) as a colorless and hygroscopic powder (348 mg, 69%): ^1H NMR (200 MHz, D_2O): δ 7.37–7.29 (m, 5 H, Ph), 4.82 (d, 1 H, $J_{1,2}$ 12.0 Hz, H-1), 4.69 and 4.61 (2 × d, 2 H, *J* 15 Hz, CH_2Ph), 4.00 (dd, 1 H, *J* 8.9 and 3.6 Hz, SCH), 3.85 (d, 1 H, *J* 12 Hz, H-6a), 3.70 (dd, 1 H, *J* 12.5 and 4.8 Hz, H-6b), 3.58–3.35 (m, 4 H, H-5, 3, 2 and 4), 3.31 (dd, 1 H, *J* 19.0 and 9.0 Hz) and 2.71 (dd, 1 H, *J* 19.0 and 3.6 Hz) (CH_2CON), 2.70 [t, 2 H, *J* 7.2 Hz, C(=NH_2)CH_2], 2.56 (t, 2 H, *J* 7.6 Hz, CH_2S), 2.0–1.8 (m, 2 H, C$H_2$$CH_2$S). ^{13}C NMR (50 MHz, D_2O): δ 182.1 (CH_2*C*ON), 180.8 (SCH*C*ON), 172.5 (C=N), 137.8, 131.7, 130.9 and 130.5 (Ph), 83.9 (C-1), 80.4 (C-5), 78.8 (C-3), 74.3 (C-2), 71.5 (C-4), 63.0 (C-6), 45.2 (*C*H_2Ph), 42.5 (SCH), 38.7 (*C*H_2CON), 34.4 [C(=NH_2)*C*H_2], 32.0 (CH_2S), 28.8 (*C*$H_2$$CH_2$S). Anal. Calcd for $C_{21}H_{30}ClN_3O_7S \cdot 1.0\ H_2O$: C, 48.32; H, 6.18; N, 8.05. Found: C, 48.24; H, 6.38; N, 8.00. HRFABMS Calcd for $C_{21}H_{30}N_3O_7S$ (M^+) 468.1806; found 468.1811.

4.3. β-Galactosylamidine (2b)

β-Galactosylamidine (**2b**) was synthesized from β-galactosylamine (**1b**) according to the same procedure as described for **2a**. The amine **1b** was prepared from galactose and ammonia according to the literature method,[29] but with modification using 7:1 MeOH–H_2O as solvent to drive the equilibrium of the α- and β-isomers in situ towards the β-form. The product **2b** was purified by ODS-S-50B (eluted with 5% MeOH) to afford pure **2b** as colorless and hygroscopic powder after lyophilization (275 mg, 55%): ^1H NMR (300 MHz, D_2O): δ 7.49–7.18 (m, 5 H, Ph), 4.80 (d, 1 H, $J_{1,2}$ 12 Hz, H-1), 4.69 and 4.60 (2 × d, 2 H, *J* 18.8 Hz, CH_2Ph), 4.0 (m, 2 H, H-6a and SCH), 3.8–3.6 (m, 5 H, H-5, 3, 2, 4 and H-6b), 3.30 (dd, 1 H, *J* 19.0 and 8.8 Hz, CH_2CON), 2.82–2.65 [m, 3 H, C(=NH_2)CH_2 and

CH_2CON], 2.59 (t, 2 H, J 7.8 Hz, CH_2S), 2.0–1.8 (m, 2 H, CH_2CH_2S). ^{13}C NMR (50 MHz, D_2O): δ 182.0 and 180.7 (C=O), 172.6 (C=N), 138.0, 131.8 131.0 and 130.7 (Ph), 84.7 (C-1), 79.8 (C-5), 75.9 (C-3), 71.8 (C-2), 71.3 (C-4), 63.7 (C-6), 45.3 (CH_2Ph), 42.7 (SCH), 38.9 (CH_2CON), 34.6 [$C(=NH_2)CH_2$], 32.4 (CH_2S), 29.0 (CH_2CH_2S). Anal. Calcd for $C_{21}H_{30}ClN_3O_7S\cdot 0.2\ H_2O$: C, 49.69; H, 6.04; N, 8.28. Found: C, 47.17; H, 6.05; N, 7.89. HRFABMS Calcd for $C_{21}H_{30}N_3O_7S$ (M^+) 468.1806; found 468.1800.

4.4. β-Glucosylamide (3)

A mixture of **1a** (359 mg, 2.0 mmol), γ-thiobutyrolactone (204 mg, 2.0 mmol), *N*-benzylmaleimide (469 mg, 2.5 mmol) and NEt_3 (405 mg, 4 mmol) in dry pyridine (4 mL) was stirred at room temperature under an argon atmosphere. The reaction was monitored by ^1H NMR. When the conversion reached 50% (after 5 days), the reaction mixture was evaporated to dryness. The residual oil was dissolved in water and washed with AcOEt. The aqueous layer was filtered through a MILLEX-GV filter (0.22 μm, Millipore) and applied to a medium-pressure ODS column (ODS-S-50B) equilibrated with water. The column was eluted with a linear gradient from water to 50% MeOH at a flow rate of 8 mL/min. The fractions containing the product (eluted at 44% MeOH) were collected and lyophilized to afford the β-glucosylamide (**3**) as colorless powder (268 mg, 28%): ^1H NMR (200 MHz, CD_3OD): δ 7.4–7.2 (m, 5 H, Ph), 4.8 (overlapped with OH, H-1), 4.64 (s, 2 H, CH_2Ph), 4.3–4.1 (m, 1 H, SCH), 4.1 (m, 1 H, H-6a), 3.7 (m, 1 H, H-6b), 3.8–2.9 [m, 10 H, H-2, 3, 4, 5, CH_2CON, $C(=O)CH_2$ and CH_2S], 2.8–2.6 (m, 2 H, CH_2CH_2S). ^{13}C NMR (50 MHz, D_2O): δ 180.3 and 179.8 (succinimide C=O), 177.3 (amide C=O), 137.3, 129.5, 129.3 and 128.7 (Ph), 91.3, 89.0, 78.8, 75.2, 74.7, 71.7, 62.8, 55.6, 53.6, 43.1, 38.5, 37.6. Anal. Calcd for $C_{21}H_{28}N_2O_8S$: C, 53.83; H, 6.02; N, 7.45. Found: C, 53.98; H, 6.02; N, 7.39.

4.5. Affinity adsorbent with β-glucosylamidine as ligand 5

TSKgel Amino TOYOPEARL 650M (dry weight, 400 mg; wet volume, 2 mL; total titrable NH_2 group, 200 μmol) was suspended in dry Me_2SO (5 mL). GMBS (448 mg, 1.6 mmol, 8 equiv of total NH_2 group) was added, and the mixture was shaken at room temperature for 10 h. The resulting suspension was filtered, and the gel was washed successively with water, EtOH and hexane, and air-dried. Unreacted amino groups were blocked by shaking the gel in a mixture of acetic anhydride (2 mL) and pyridine (2 mL) for 3 h. The resulting gel **4** was filtered and washed successively with water, EtOH and hexane. To a suspension of the gel **4** in dry pyridine (5 mL) were added β-glucosylamine (**1a**) (180 mg, 1 mmol) and 2-iminothiolane·HCl (138 mg, 1 mmol) at room temperature. The mixture was shaken at room temperature for 24 h and filtered. The gel was washed successively with water, EtOH and hexane. The unreacted maleimido group was blocked by shaking the gel with 2-mercaptoethanol (340 μL, 5 mmol) in dry pyridine (5 mL) at room temperature for 4 h. The gel was filtered and washed successively with water, EtOH and hexane, and air-dried. The resulting gel **5** was suspended in 0.1 M sodium citrate buffer (pH 6) and stored in a refrigerator.

The moles of the ligand was calculated from the moles of the amino groups of the matrix before and after the modification with GMBS. The gel (dry weight, 218 mg; wet volume, 1.5 mL) was suspended in Me_2SO (5 mL), and *p*-nitrophenyl acetate (906 mg, 5 mmol) was added. The mixture was shaken at room temperature for 2.5 h. An aliquot of the supernatant was taken and was diluted with 50 mM Tris–HCl (pH 8.0). The concentration of *p*NP was determined by measuring the absorbance at 405 nm. The amount of amino group was calculated as 88 and 1.9 μmol/mL gel (wet volume) before and after modification, respectively, corresponding to 98% modification rate.

A control gel **6** without β-glucosylamidine ligand was prepared by adding 2-mercaptoethanol (340 μL, 5 mmol) directly to the gel **4**.

4.6. Affinity adsorbent with β-glucosylamidine (8a)

TSKgel Carboxy TOYOPEARL 650M (dry weight, 700 mg; wet volume, 3.5 mL; total titrable COOH group, 350 μmol) was allowed to react with *N*-hydroxysuccinimide (202 mg, 1.8 mmol, 5 equiv) and EDC (337 mg, 1.8 mmol, 5 equiv) in dioxane (12 mL) at room temperature. After shaking for 5 min, 4,7,10-trioxatridecane-1,13-diamine (771 mg, 3.5 mmol) was added to the suspension. After shaking at room temperature for 12 h, the gel was filtered and washed successively with water, EtOH and hexane, and air-dried. Unreacted carboxyl groups were blocked by reacting with EDC (337 mg, 1.8 mmol) and ethanolamine (110 mg, 1.8 mmol) in dioxane (10 mL) for 1 h. The gel was filtered and washed successively with water, EtOH and hexane. The resulting gel was allowed to react with GMBS (490 mg, 1.8 mmol) in dry Me_2SO (5 mL) at room temperature for 5 h. The unreacted amino groups were blocked with acetic anhydride (3 mL) and pyridine (2 mL) as described above. The resulting gel **7** was allowed to react with β-glucosylamine (**1a**) (315 mg, 1.8 mmol) and 2-iminothiolane·HCl (242 mg, 1.8 mmol) in dry pyridine (10 mL). After shaking at room temperature for 12 h, the gel was filtered and washed. The unreacted maleimido groups were blocked with 2-mercaptoethanol as described above. The gel **8a** was suspended in 0.1 M sodium citrate buffer (pH 6) and was stored in a refrigerator.

4.7. Affinity adsorbent with β-galactosylamidine (8b)

The gel **7** (dry weight, 400 mg; wet volume, 2 mL) was allowed to react with β-galactosylamine (**1b**) (72 mg, 0.4 mmol) and 2-iminothiolane·HCl (55 mg, 0.4 mmol) in dry pyridine (2 mL). After shaking the mixture at room temperature for 12 h, the gel was filtered and washed. The unreacted maleimide groups were blocked with 2-mercaptoethanol as described above. The gel **8b** was recovered and stored in refrigerator as a suspended in 0.1 M sodium citrate buffer (pH 6).

4.8. Enzyme assay

A typical method for measuring enzyme activity is as follows: an appropriate amount of β-glucosidase (50 μL) in 50 mM sodium acetate buffer (pH 5.0) containing 200 μg/mL BSA was added to a preincubated solution (950 μL) of *p*NP-β-Glc (0.3–1.2 mM) in 50 mM sodium acetate buffer (pH 5.0) at 30 °C (total volume of 1 mL). The mixture was incubated at 30 °C for 10 min. The reaction was terminated by adding 1 M Na_2CO_3 solution (500 μL), and the absorbance at 405 nm was measured. The initial rate (v_0) was calculated using ε (*p*NP) = 17.8 cm^2/μmol. For *o*NP-glycosides, the absorbance at 420 nm was measured, and the initial rate (v_0) was calculated using ε (*o*NP) = 4.44 cm^2/μmol.

The following glycosidases and assay conditions (substrate, buffer, pH, temperature and K_m) were used for the inhibition experiment: (1) β-glucosidase from *A. niger*, chromatography purified (a gift from Dr Unno, Nihon Shokuhin Kako Co., Ltd.) [*p*NP-β-Glc, 50 mM sodium acetate buffer (pH 5.0), 30 °C, K_m = 0.61 mM]. (2) β-Glucosidase from *T. viride*, chromatography purified (a gift from Dr Okada, Shizuoka University, Japan) [*p*NP-β-Glc, 50 mM sodium acetate buffer (pH 5.0), 30 °C, K_m = 0.11 mM]. (3) β-Glucosidase from almond, chromatography purified (Sigma, cat. No. G4511) [*p*NP-β-Glc, 50 mM sodium acetate buffer (pH 5.0), 30 °C, K_m = 2.37 mM]. (4) β-Galactosidase from *Aspergillus oryzae* (Sigma, cat. No. G7138) [*o*NP-β-Gal, 50 mM sodium acetate buffer (pH 5.0), 30 °C, K_m = 0.97 mM]. (5) β-Galactosidase from *Escherichia coli* (Sigma, cat. No. G5635, Grade VIII) [*o*NP-β-Gal, 50 mM sodium phosphate buffer (pH 7.0), 30 °C, K_m = 0.24 mM]. (6) β-Xylosidase from *Aspergillus pulverulentus* (Amano Pharmaceutical Co., Ltd.) [*p*NP-β-Xyl, 50 mM sodium acetate buffer (pH 4.0), 30 °C, K_m = 0.21 mM]. (7) β-Glucosidase from tea leaves (affinity purified) [*p*NP-β-Glc, 20 mM sodium citrate buffer (pH 4.5), 37 °C, K_m = 0.70 mM]. (8) β-Galactosidase from *P. multicolor* (affinity purified) [*o*NP-β-Gal, 20 mM sodium acetate buffer (pH 5.0), 37 °C, K_m = 0.46 mM].

The activity of fractions of affinity chromatography of tea leaf β-glucosidase was measured using 10 mM *p*NP-β-Glc in 20 mM sodium citrate buffer (pH 6.0) at 37 °C for 5 min. The activity of fractions of *P. multicolor* β-galactosidase was measured using 5 mM *o*NP-β-Gal in 20 mM sodium citrate buffer (pH 4.0) at 37 °C for 5 min. One unit of enzyme activity was defined as the amount of enzyme that catalyzed the formation of 1 μmol of *p*NP or *o*NP per minute at 37 °C.

For determining the inhibition constant (K_i), the reaction was initiated by adding an enzyme solution (50 μL) to an assay mixture containing the substrate (the concentrations were equal to or twice the K_m value for each enzyme) and varying concentrations of the inhibitor, and the enzyme activity was measured. The type of inhibition was analyzed by double-reciprocal plot and was found to be competitive with respect to the substrate. The inhibition constants (K_i) were determined by a Dixon plot.

4.9. Protein assay

Protein concentrations were determined by the method of Bradford[44] using BSA as the reference protein.

4.10. Affinity chromatography

4.10.1. Preparation of crude enzyme extract from tea leaves. Tea leaf acetone powder was prepared from freshly plucked tea leaves (*Camellia sinensis* var. *sinensis* cv. Yabukita) according to the reported procedure.[35] Enzymes were extracted from 100 g of the acetone powder with 2 L of 0.1 M sodium citrate buffer (pH 6) by stirring at 4 °C for 4 h. The resulting suspension was filtered roughly with sterilized gauze to remove the residues, and the filtrate was centrifuged at 14,000*g* at 4 °C for 30 min. To the supernatant, an equal volume of chilled acetone (−20 °C) was added gradually, and the mixture was stirred at 0 °C for 1 h and at 4 °C overnight. The mixture was centrifuged at 14,000*g* at 4 °C for 10 min. The precipitate was suspended in 500 mL of 0.1 M sodium citrate buffer (pH 6), and the mixture was stirred at 0 °C for 1 h. The suspension was centrifuged at 14,000*g* at 4 °C for 10 min, and the supernatant was used as crude enzyme solution for affinity chromatography.

4.10.2. Affinity purification of β-glucosidase from tea leaves. Each of the following steps was performed at 4 °C unless otherwise noted. The control gel **6** without ligand (wet volume of 1.2 mL) and the affinity adsorbent **5** (wet volume of 2 mL) were placed each in a plastic column (Poly-Prep™ Chromatography Column, 0.8 × 4 cm, Bio-Rad), and each column was washed and equilibrated with 0.1 M sodium citrate buffer (pH 6). A crude enzyme solution (0.32 mg protein/mL, 10 mL) was applied to the control gel **6** with a flow rate of 0.1 mL/min to obtain the pass-through fraction (10 mL, Fr.

1) with high β-glucosidase activity. The Fr. 1 was applied to the affinity adsorbent **5** with a flow rate of 0.05 mL/min to give a pass-through fraction (10 mL, Fr. 2). The column was washed with 20 mL of 0.1 M sodium citrate buffer (pH 6) with a flow rate 0.15 mL/min to remove loosely bound substances from the adsorbent. The eluent was fractioned and collected (2 mL each, Fr. 3–10). The adsorbent was washed with 10 mL of 1 M NaCl in 0.1 M sodium citrate buffer (pH 6) with a flow rate of 0.13 mL/min, and 2.5 mL-fractions were collected (Fr. 11–15). The column was eluted with 0.5 M D-glucose in 0.1 M sodium citrate buffer (pH 6) with a flow rate of 0.1 mL/min, and 2 mL-fractions were collected (Fr. 16–21). Finally, the column was washed with 5 M urea in H_2O with a flow rate 0.17 mL/min, and 2.5 mL-fractions were collected (Fr. 22–25). The protein content and the enzyme activity of each fraction were determined by measuring the absorbance at 280 nm and β-glucosidase activity with *p*NP-β-Glc as substrate.

4.10.3. Affinity purification of β-galactosidase from *P. multicolor*. Each of the following steps was performed at 4 °C unless otherwise noted. The affinity adsorbent **8b** (wet volume of 2 mL) was placed in a plastic column and washed with 20 mL of 20 mM sodium acetate buffer (pH 5). A crude enzyme solution from *P. multicolor* [0.43 mg protein/mL in 20 mM sodium acetate buffer (pH 5), 1 mL] was applied to the column with a flow rate of 0.1 mL/min. Elution of β-galactosidase was performed with 0–250 mM NaCl gradient, and the eluent was fractionated (2 mL each, Fr 1–38). Each fraction was assayed for protein content (absorbance at 280 nm) and β-galactosidase activity with *o*NP-β-Gal as substrate.

4.11. Sodium dodecyl sulfate polyacrylamide gel electrophoresis

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was done by the reported procedure.[45] The following molecular weight markers (BioLabs) was used (subunit *M*r is shown in parentheses): myosin (212,000), MBP-β-galactosidase (158,194), β-galactosidase (116,351), phosphorylase b (97,184), BSA (66,409), glutamic dehydrogenase (55,561), MBP 2 (42,710), lactate dehydrogenase M (36,487), triosephosphate isomerase (26,625), trypsin inhibitor (20,100), lysozyme (14,313), aprotinin (6517), insulin (2340–3400). Protein bands were detected by silver staining according to Merril and co-workers[46] using a Silver Stain Kit Wako (Wako Pure Chemical Industries, Ltd., Japan).

Acknowledgements

We thank Amano Enzyme Inc. for a generous gift of β-galactosidase from *Penicillium multicolor*. This study was supported in part by a Grant-in-Aid for Scientific Research from Japan Society for the Promotion of Science (contract # 13480187 and 13460049) and by a Grant-in-Aid from the Ministry of Education, Culture, Sports, Science and Technology, Japan (21COE on Kyoto University Alliance for Chemistry).

References

1. Dwek, R. A. *Chem. Rev.* **1996**, *96*, 683–720.
2. Kornfeld, R.; Kornfeld, S. *Annu. Rev. Biochem.* **1985**, *54*, 631–664.
3. Henrissat, B. *Biochem. J.* **1991**, *280*, 309–316.
4. Henrissat, B.; Davies, G. *Curr. Opin. Struct. Biol.* **1997**, *7*, 637–644.
5. Wilchek, M.; Miron, T.; Kohn, J. *Methods Enzymol.* **1984**, *104*, 3–55.
6. Barker, R.; Chiang, C.-K.; Trayer, I. P.; Hill, R. L. *Methods Enzymol.* **1974**, *34*, 317–328.
7. Giudicelli, J.; Emiliozzi, R.; Vannier, C.; De Burlet, G.; Sudaka, P. *Biochim. Biophys. Acta* **1980**, *612*, 85–96.
8. Grabowski, G. A.; Dagan, A. *Anal. Biochem.* **1984**, *141*, 267–279.
9. Hiraizumi, S.; Spohr, U.; Spiro, R. G. *J. Biol. Chem.* **1994**, *269*, 4697–4700.
10. Sangseethong, K.; Penner, M. H. *Carbohydr. Res.* **1998**, *314*, 245–250.
11. Mega, T.; Matsushima, Y. *J. Biochem.* **1976**, *79*, 185–194.
12. Jain, R. S.; Binder, R. L.; Levy-Benshimol, A.; Buck, C. A.; Warren, L. *J. Chromatogr.* **1977**, *139*, 283–290.
13. Piyachomkwan, K.; Gable, K. P.; Penner, M. H. *Carbohydr. Res.* **1997**, *303*, 255–259.
14. Abo, S.; Ciccotosto, S.; Alafaci, A.; von Itzstein, M. *Carbohydr. Res.* **1999**, *322*, 201–208.
15. Watanabe, T.; Sato, T.; Yoshioka, S.; Koshijima, T.; Kuwahara, M. *Eur. J. Biochem.* **1992**, *209*, 651–659.
16. Harpaz, N.; Flowers, H. M.; Sharon, N. *Biochim. Biophys. Acta* **1974**, *341*, 213–221.
17. Harpaz, N.; Flowers, H. M. *Methods Enzymol.* **1974**, *34*, 347–350.
18. Harpaz, N.; Flowers, H. M.; Sharon, N. *Eur. J. Biochem.* **1977**, *77*, 419–426.
19. Bishop, D. F.; Desnick, R. J. *J. Biol. Chem.* **1981**, *256*, 1307–1316.
20. Presper, K. A.; Concha-Slebe, I.; De, T.; Basu, S. *Cabohydr. Res.* **1986**, *155*, 73–87.
21. Hettkamp, H.; Legler, G.; Bause, E. *Eur. J. Biochem.* **1984**, *142*, 85–90.
22. Kang, M. S.; Liu, P. S.; Bernotas, R. C.; Harry, B. S.; Sunkara, P. S. *Glycobiology* **1995**, *5*, 147–152.
23. Legler, G.; Bieberich, E. *Arch. Biochem. Biophys.* **1988**, *260*, 427–436.
24. Matern, H.; Heinemann, H.; Legler, G.; Matern, S. *J. Biol. Chem.* **1997**, *272*, 11261–11267.
25. Bause, E.; Gross, A.; Schweden, J. *FEBS Lett.* **1991**, *278*, 167–170.
26. Schweden, J.; Bause, E. *Biochem. J.* **1989**, *264*, 347–355.

27. Scudder, P.; Neville, D. C. A.; Butters, T. D.; Fleet, G. W.; Dwek, R. A.; Rademacher, T. W.; Jacob, G. S. *J. Biol. Chem.* **1990**, *265*, 16472–16477.
28. Guo, W.; Hiratake, J.; Ogawa, K.; Yamamoto, M.; Ma, S.-J.; Sakata, K. *Bioorg. Med. Chem. Lett.* **2001**, *11*, 467–470.
29. Isbell, H. S.; Frush, H. L. *J. Org. Chem.* **1958**, *23*, 1309–1319.
30. Morrison, J. F.; Walsh, C. T. *Adv. Enzymol. Rel. Areas Mol. Biol.* **1988**, *61*, 201–301.
31. Schloss, J. V. *Acc. Chem. Res.* **1988**, *21*, 348–353.
32. Bülow, A.; Plesner, I. W.; Bols, M. *Biochim. Biophys. Acta* **2001**, *1545*, 207–215.
33. Cuatrecasas, P.; Anfinsen, C. B. *Ann. Rev. Biochem.* **1971**, *40*, 259–278.
34. Steers, E., Jr.; Cuatrecasas, P. *Methods Enzymol.* **1974**, *34*, 350–358.
35. Ijima, Y.; Ogawa, K.; Watanabe, N.; Usui, T.; Ohnishi-Kameyama, M.; Nagata, T.; Sakata, K. *J. Agric. Food Chem.* **1998**, *46*, 1712–1718.
36. Manger, I. D.; Rademacher, T. W.; Dwek, R. A. *Biochemistry* **1992**, *31*, 10724–10732.
37. Dale, M. P.; Ensley, H. E.; Kern, K.; Sastry, K. A. R.; Byers, L. D. *Biochemistry* **1985**, *24*, 3530–3539.
38. He, S.; Withers, S. G. *J. Biol. Chem.* **1997**, *272*, 24864–24867.
39. Esen, A. β-Glucosidases: Overview; Conn, E.E. β-Glucosidases in Plants: Substrate Specificity. In: Esen, A. (Ed.) *β-Glucosidases. Biochemistry and Molecular Biology*, ACS Symposium series 533: ACS, Washington, DC, 1994; pp. 1–14 and 15–26.
40. Czjzek, M.; Cicek, M.; Zamboni, V.; Bevan, D. R.; Henrissat, B.; Esen, A. *Proc. Natl. Acad. Sci. USA* **2000**, *97*, 13555–13560.
41. Unno, T.; Ide, K.; Yazaki, T.; Tanaka, Y.; Nakakuki, T.; Okada, G. *Biosci. Biotech. Biochem.* **1993**, *57*, 2172–2173.
42. Narayanan, S. R. *J. Chromatogr. A* **1994**, *658*, 237–258.
43. Rich, D. H.; Gesellchen, P. D.; Tong, A.; Cheung, A.; Buckner, C. K. *J. Med. Chem.* **1975**, *18*, 1004–1010.
44. Bradford, M. M. *Anal. Biochem.* **1976**, *72*, 248–254.
45. Laemilli, U. K. *Nature* **1970**, *227*, 680–685.
46. Merril, C. R.; Dunan, M. L.; Godman, D. *Anal. Biochem.* **1981**, *110*, 201–207.