Biosci. Biotechnol. Biochem., **64** (8), 1702–1706, 2000

Hydrolysis of β-Galactosyl Ester Linkage by β-Galactosidases

Taro KISO,[1] Hirofumi NAKANO,[1] Hirofumi NAKAJIMA,[2] Tadamasa TERAI,[2] Katsuyuki OKAMOTO,[3] and Sumio KITAHATA[1]

[1]*Osaka Municipal Technical Research Institute, 1-6-50 Morinomiya, Joto-ku, Osaka 536-8553, Japan*
[2]*Osaka Institute of Technology, 5-16-1, Ohmiya, Asahi-ku, Osaka 535-8585, Japan*
[3]*Showa sangyo Co., Ltd., 1-16, Sakura, Tsukubashi, Ibaraki 350-0003, Japan*

Received February 17, 2000; Accepted March 28, 2000

p-Hydroxybenzoyl β-galactose (*p*HB-Gal) was synthesized chemically to examine the hydrolytic activity of β-galactosyl ester linkage by β-galactosidases. The enzyme from *Penicillium multicolor* hydrolyzed the substrate as fast as *p*-nitrophenyl β-galactoside (*p*NP-Gal), a usual substrate with a β-galactosidic linkage. The enzymes from *Escherichia coli* and *Aspergillus oryzae* hydrolyzed *p*HB-Gal with almost the same rates as *p*NP-Gal. The enzymes from *Bacillus circulans*, *Saccharomyces fragilis*, and bovine liver showed much lower activities. pH-activity profiles, inhibition analysis, and kinetic properties of the enzymic reaction on *p*HB-Gal suggested that β-galactosidase had only one active site for hydrolysis of both galactosyl ester and galactoside. The *Penicillium* enzyme hydrolyzed *p*HB-Gal in the presence of $H_2{}^{18}O$ to liberate galactose containing ^{18}O. This result suggests the degradation occurs between the anomeric carbon and an adjacent O atom in the ester linkage of *p*HB-Gal.

Key words: β-galactosidase; galactosyl ester linkage; active site

β-Galactosidase (EC 3.2.1.23), which hydrolyzes β-galactosidic linkages of oligosaccharides and galactosides, is one of the most characterized enzymes and has practical importance, for example, in the production of low lactose-containing milk or synthesis of galactooligosaccharides.

We previously reported that some β-glucosidases can cleave β-glucosyl ester linkages of steviol glycosides.[1,2] However, hydrolysis of other glycosyl esters by glycosidases remains unknown. It is interesting to investigate hydrolysis of β-galactosyl esters by β-galactosidase. Nevertheless, it has not been reported whether β-galactosidases can hydrolyze β-galactosyl esters because there are no β-galactosyl esters among natural compounds. In this study *p*-hydroxybenzoyl β-galactose (*p*HB-Gal, Fig. 1) was chemically synthesized to investigate the hydrolytic properties of β-galactosidase on the β-galactosyl ester and the detailed features of hydrolysis of the galactosyl ester by some β-galactosidases were studied.

Materials and Methods

Chemicals. *p*-Acetoxybenzoic acid was purchased from Tokyo Kasei Co. *p*-Nitrophenyl β-galactoside (*p*NP-Gal), acetobromogalactose, and isopropyl β-thio-galactoside (IPTG) were purchased from Sigma Chemical Co. Other chemicals were from Nacalai Tesque Co. β-1,4-Linked galactobiose (Gal_2) was synthesized as in a previous report.[3] The octadecylsilyl (ODS) resin used for adsorption of *p*HB-Gal was an HLB 1 cc Extraction Cartridge from Waters Corporation.

Synthesis of pHB-Gal. *p*HB-Gal was synthesized after Schmidt and Reuss,[4] who reported synthesis of the glucosyl ester. *p*-Acetoxybenzoic acid (*p*ABA; 44 g, 0.24 mol) was dissolved in 80% acetone (222 ml) and was neutralized with 3 N KOH to pH 7.0. Acetobromo α-D-galactose (50 g, 0.12 mol) in acetone (61 ml) was added to the *p*ABA solution. The mixture was left at room temperature for 5 h with stirring and then at −20°C overnight. The white crystals of *p*HBA were eliminated by filtration, and evaporation of the filtrate provided a viscous materi-

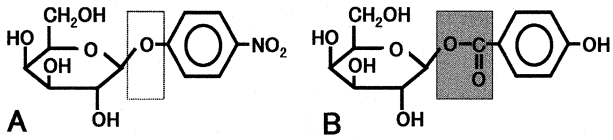

Fig. 1. Structure of Substrates.
*p*NP-Gal (A) has β-galactoside linkage in the dotted box. *p*HB-Gal (B) is a β-galactosyl ester of *p*-hydroxybenzoic acid. The gray-colored box emphasizes β-galactosyl ester linkage.

To whom correspondence should be addressed. Taro KISO, FAX: +81-6-6963-8079; E-mail: tkiso@omtri. city.osaka.jp

Abbreviations: *p*ABA, *p*-acetoxybenzoic acid; *p*HB-Gal, *p*-hydroxybenzoyl β-galactose; *p*NP-Gal, *p*-nitrophenyl β-galactoside; *p*HBA, *p*-hydroxybenzoic acid; *p*NP, *p*-nitrophenol; IPTG, isopropyl β-thio-galactoside; Gal_2, β-1,4-linked galactobiose

al (*p*-acetoxybenzoyl tetra-*O*-acetyl-*β*-galactoside). This viscous material was dissolved in methanol (54 ml) and deacetylated with 4.0 M CH_3ONa in methanol (80 ml). Deacetylation was stopped by application of this solution to an Amberlite IR-120 column (100 ml). The methanol eluate was evaporated and the resulting brownish material was solved in 100 ml of deionized water. *p*ABA non-reacted was eliminated by extraction with ethyl acetate. The water phase was applied to an active carbon column. Pure *p*HB-Gal was obtained by evaporation of the 35% ethanol eluate.

Enzyme. *β*-Galactosidases originated from bovine liver, *Escherichia coli*, and *Sccharomyces fragilis* were purchased from Sigma Chemical Co. Enzyme of *Aspergillus oryzae* was from Yakult Co. Ltd. Daiwa Kasei Co. Ltd. supplied *β*-galactosidase from *Bacillus circulans*, Kei-Ai Kasei Co. did one from *Penicillium multicolor*. *β*-Galactosidases from the *A. oryzae*, *B. circulans*, bovine liver, *E. coli*, and *S. fragilis* were purified by extraction from polyacrylamide gel after Native PAGE. *P. multicolor*[5] enzyme was purified by sequential application to Q-Sepharose, Sephacryl S-100HR, and Hydroxy apatite columns into a homogeneous state observed by SDS-PAGE.

Enzyme assay. Enzymes were diluted by appropriate buffers. Phosphate buffer at pH 7.0 was used for enzyme from bovine liver, *E. coli*, and *S. fragilis*. *β*-Galactosidases of the *A. oryzae*, *B. circulans*, and *P. multicolor* were diluted with phosphate buffer (pH 6.0).

For investigation of pH-activity profile, buffers used were 50 mM H_3PO_4–Na_2HPO_4 (pH 3–5.5) and 50 mM Na_2HPO_4–KH_2PO_4 (pH 5.6–8).

*p*NP-Gal solution (250 μl) and enzyme solution (250 μl) were mixed and incubated at 40°C for 10 min. The reaction was stopped by the addition of 0.2 M Na_2CO_3 (500 μl). Hydrolytic activity on *p*NP-Gal was determined by the liberation of *p*NP.

Hydrolysis of *p*HB-Gal was measured from *p*HBA liberated. *p*HB-Gal solution (100 μl) and enzyme solution (100 μl) were mixed and were incubated at 40°C for 10 min. Addition of 0.1 N HCl (250 μl) stopped the enzymic reaction and the solution was neutralized with 0.1 N NaOH (250 μl). *p*HBA in the neutralized solution was measured by the 1-ethyl-3-(3-dimethylaminopropyl) carbodiimido (EDC) method,[6] which is used to measure carbonic acid.

Analytical methods for structure elucidation of pHB-Gal. *p*HB-Gal (20 mg) was solved in 600 μl of D_2O. ^{13}C-NMR spectrum was obtained with JNM-AL300 (JEOL). For infrared spectrometry *p*HB-Gal was dispersed in a KBr tablet. Then the spectrum was obtained for the tablet by Impact 400 M (Nicolet). FAB-mass spectrometry was done on hydrolytic products. The apparatus was JMS-AX505HA (JEOL).

Kinetic feature of single active site. If an enzyme reacts with two substrates, A and B, at a single active site, the scheme of the reaction for the mixed substrates of A and B is expressed as follows:

$$E + A + B \rightleftharpoons \begin{matrix} EA \longrightarrow E + P_A \\ EB \longrightarrow E + P_B \end{matrix}$$

In the above scheme the enzyme is referred to E, and the products from A and B are presented as P_A and P_B, respectively. Then the initial velocities, v_A and v_B, of the reaction are calculated as follows:

$$v_A = (k_A / K_A)[E][A].$$
$$v_B = (k_B / K_B)[E][B].$$

[E] is concentration of a free enzyme. K_A and K_B are the inverses of K_m values observed for pure substrates A and B, respectively. k_A and k_B express k_{cat} values for the pure substrates A and B. The kinetic behavior on the mixed substrate of A and B follows the below formula,[7] only when the enzyme has only one active site by dividing the above v_A by the v_B.

$$v_A / v_B \propto [A]/[B].$$

If enzyme has more than two active sites, there appear cross terms of [A] and [B] like following formula.

$$v_A = [E]\{a[A] + b[B] + c[A][B]\}$$
$$v_B = [E]\{a'[A] + b'[B] + c'[A][B]\}$$

where a, a′, b, b′, c, and c′ present constant values related to K_A, K_B, k_A, or k_B. Therefore, the v_A/v_B is not proportional to [A]/[B] in general.

Results and Discussion

Structure of synthesized pHB-Gal

Although a proper substrate was looked for in order to investigate hydrolytic activity on galactosyl ester of *β*-galactosidase, no natural compound having *β*-galactosyl ester linkage has been discovered. In this study, we synthesized an artificial substrate, *p*-hydroxybenzoyl *β*-D-galactose (*p*HB-Gal). Because synthesis of *p*HB-Gal has not been reported previously, structure of the product was checked somewhat in detail by FT-IR and ^{13}C-NMR.

Infrared spectrum of *p*HB-Gal also demonstrated galactosyl ester linkage. Carboxyl moiety of *p*HBA absorbed at 931, 1317, and 1420 cm^{-1}, while absorbance of this wave numbers were not observed for *p*HB-Gal. Instead absorbance at 1216 and 1077 cm^{-1} for *p*HB-Gal was attributed to ester linkage of aromatic acid. *p*HBA absorbed at 1686 cm^{-1} for C=O double bond, alternatively *p*HB-Gal absorbed at

1707 cm^{-1} for C=O of ester linkage.

^{13}C-NMR spectrum (Table 1) of *p*HB-Gal had carbon signals of galactosyl groups, C-2, 3, 4, 5, and 6, which corresponded to each of the galactose residues, respectively. A signal at δ 95.2 ppm was attributed to an anomeric carbon (C-1). This signal showed a chemical shift near that of anomeric carbon of steviol glycoside (95.7 ppm),[8] which had a β-type of glucosyl ester linkage. It was, therefore, confirmed that this synthesized substrate was a β-galactosyl ester. Comparison with spectrum on *p*HBA assigned the other four signals with signals of C′-1 to 7 in *p*-hydroxy benzoyl group. The signal of C′-7 (δ 167.1) of *p*HB-Gal was observed at a higher field than that of carbonyl carbon of *p*HBA by 2.2 ppm. This shift resulted from the carbonyl carbon of the *p*-hydroxybenzoyl group participating in linkage with the galactosyl moiety. Consequently the synthesized *p*HB-Gal was found to have expected structure (Fig. 1).

Course of hydrolysis of pHB-Gal

Figure 2 displays the course of the hydrolysis of *p*HB-Gal by β-galactosidase originated from *E. coli*. Galactose was measured by the Somogyi-Nelson method. *p*HBA liberated was measured by the EDC method.[6] *p*HB-Gal remaining in the reaction mixtures was removed by adsorption on ODS resin, because *p*HB-Gal prevented normal coloring. *p*HBA was liberated at an equal molar amount with galactose through the time tested. In the following study, therefore, hydrolytic activities of *p*HB-Gal by β-galactosidases could be measured by liberation of *p*HBA by the EDC method.

Hydrolytic activities of some β-galactosidases for pHB-Gal

Hydrolytic activities of β-galactosidases for *p*HB-Gal (2 mM) were shown as relative activities against those for *p*NP-Gal (2 mM) in Fig. 3. Enzymes from *E. coli* and *P. multicolor* displayed relative activities of about 1.0 under the reaction conditions adopted. This result shows that these enzymes hydrolyzed *p*HB-Gal as easily as *p*NP-Gal, which was generally known to be a more favorable substrate than lactose, a natural substrate for β-galactosidases.[5] Therefore, the activities against *p*HB-Gal are considered to be high enough and well worthy studying.

Hydrolytic activity of *Aspergillus* β-galactosidase for *p*HB-Gal was about 80% of that for *p*NP-Gal. Bovine liver enzyme displayed a little activity on *p*HB-Gal. On the other hand, *S. fragilis* and *B. circulans* β-galactosidases cleaved the β-galactosyl ester linkage much less than the β-galactosidic linkage. The enzymes from different origines presented the different activities on *p*HB-Gal. It is supposed that the specific activity on *p*HB-Gal of each enzyme reflected structural characteristics of its active site. In the following experiments β-galactosidases from *E. coli*, *P. multicolor*, and *A. oryzae* were used because of their high activities on *p*HB-Gal.

Table 1. Chemical Shifts Obtained by ^{13}C-NMR of *p*HB-Gal

Position of carbon	Chemical shift (ppm)
C-1	95.2
2	70.0
3	72.9
4	68.8
5	76.5
6	61.1
C-1′	120.3
2′,6′	132.6
3′,5′	115.9
4′	161.8
7′	167.1

The solvent was D_2O.

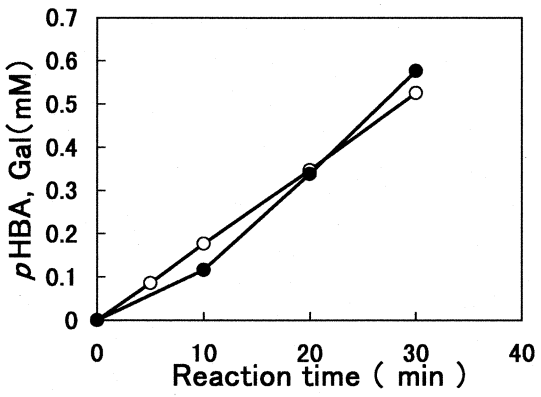

Fig. 2. Course of Hydrolysis of *p*HB-Gal by *E. coli* β-Galactosidase.

The concentration of *p*HB-Gal was 2.0 mM and reaction was done at 40°C and in the phosphate buffer (pH 7.0). Liberation of *p*HBA (○) was measured by EDC method. Liberation of galactose (●) was measured by the Somogyi-Nelson method after removal of *p*HB-Gal with ODS resin.

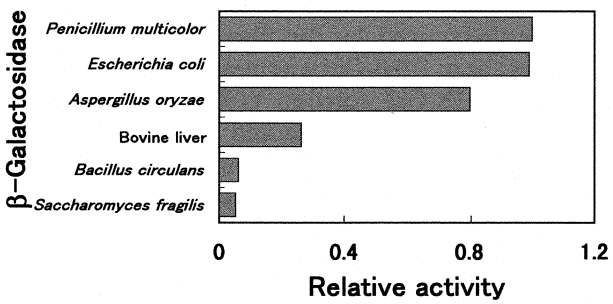

Fig. 3. Relative Activities of Several β-Galactosidases for *p*HB-Gal against *p*NP-Gal.

Hydrolytic activities toward *p*HB-Gal were divided by those toward *p*NP-Gal. Enzymes were from *Penicillium multicolor*, *Escherichia coli*, *Aspergillus oryzae*, bovine liver, *Bacillus circulans*, and *Saccharomyces fragilis*. The reaction mixture contained 2.0 mM substrate. The reaction was done at 40°C for 10 min.

pH-activity profile

To investigate the hydrolysis of galactosyl ester more precisely, the pH-dependency of enzymatic reaction on *p*HB-Gal was analyzed (Fig. 4). pH-activity profile on *p*NP-Gal was also measured for comparison. *p*HB-Gal and *p*NP-Gal were hydrolyzed at pH 3.0~8.0 by β-galactosidases from *E. coli* and *P. multicolor*. Buffers used are shown in "Materials and Methods".

The optimum pH of *Penicillium* enzyme for cleavage of the galactosyl ester was 4~5 like that for hydrolysis of *p*NP-Gal. Optimum pH of *E. coli* for *p*HB-Gal hydrolysis coincided with that for *p*NP-Gal hydrolysis at the range of 7.0. On both enzymes, the profile of activity for *p*HB-Gal drew similar curves to that for *p*NP-Gal. Change of pH gives rise to alteration of dissociation of active residues in the enzyme.[9] Similarity of the pH-activity profile and optimum pH for both substrates, therefore, suggested a resemblance of environment of the active center.

Inhibition analysis of hydrolysis of pHB-Gal

To obtain detailed information for cleavage of *p*HB-Gal, we investigated the hydrolysis of *p*HB-Gal and *p*NP-Gal in the presence of two inhibitors, β-1,4-linked galactobiose (Gal_2) and isopropyl β-thiogalactopyranoside (IPTG). It has been reported that Gal_2 was a competitive inhibitor for *E. coli* β-galactosidase.[3] The enzyme used here was originated from *E. coli*. S *vs.* S/*v* plots in hydrolysis of these substrates showed parallel straight lines with increasing concentrations of IPTG and Gal_2 (data not shown). These results suggest these inhibitors were as competitive on hydrolysis of both *p*HB-Gal and *p*NP-

Table 2. Type of Inhibition by IPTG and Gal_2 toward Hydrolysis of *p*HB-Gal and *p*NP-Gal

Inhibitor	Substrate	K_i (mM)	Type of inhibition
Gal_2	*p*HB-Gal	0.03	Competitive
	*p*NP-Gal	0.07	Competitive
IPTG	*p*HB-Gal	0.17	Competitive
	*p*NP-Gal	0.29	Competitive

The reaction was done at 40°C for 10 min. K_i was calculated from secondary plot of K_m/V_{max} under inhibition.

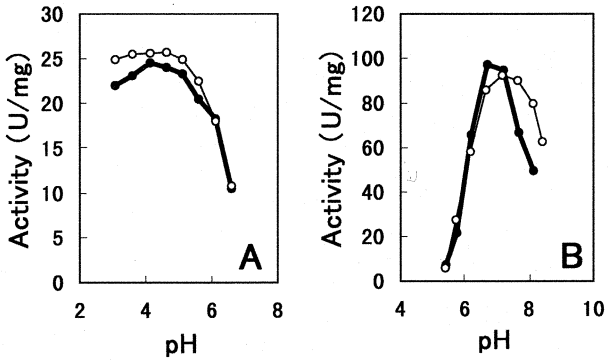

Fig. 4. pH-Activity Profile of Hydrolysis toward *p*HB-Gal.

*p*HB-Gal, hollow circles and thin lines; *p*NP-Gal, solid circles and thick lines. Enzymes used were β-galactosidases from *P. multicolor* (A) and *E. coli* (B). Reaction was at 40°C for 10 min and concentration of substrate was 8 mM.

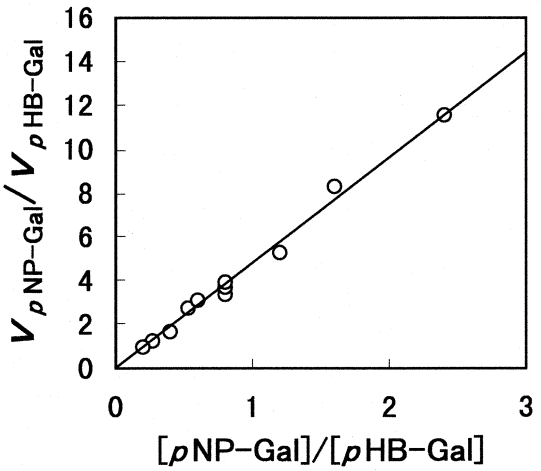

Fig. 5. Ratio of Hydrolytic Activities for Mixed Substrates of *p*NP-Gal and *p*HB-Gal.

β-Galactosidase from *E. coli* was used and reaction was done at 40°C for 10 min. Ratio of activity on *p*HB-Gal against that on *p*NP-Gal was plotted toward ratio of concentration of the two compounds (*p*HB-Gal and *p*NP-Gal) in a given mixed substrate.

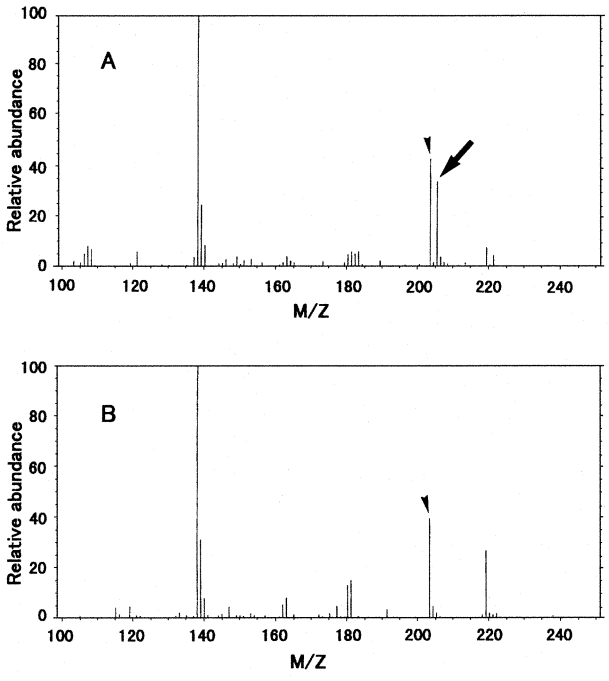

Fig. 6. FAB-MS Spectra for Hydrolysates of *p*HB-Gal by *E. coli* β-Galactosidase.

Reaction mixtures, containing *p*HB-Gal (2.0 mM) and *E. coli* enzyme (70U), were incubated at 40°C for 30 min. Reaction was done with a solvent including 50 mol% $H_2{}^{18}O$ (A) and with a solvent including no additive $H_2{}^{18}O$ (B). The arrowheads point the signal (M/Z 203) of the sodium and galactose without ^{18}O. The arrow in (A) shows the peak (M/Z 205) of the sodium and galactose containing ^{18}O.

Gal. From secondary plots of apparent K_m/V_{max}, K_i values were calculated as a function of inhibitor concentration. As summarized in Table 2, the K_i value of Gal_2 for *p*HB-Gal hydrolysis (0.03 mM) was similar with that for *p*NP-Gal (0.07 mM). Competitive inhibition by Gal_2 was observed for hydrolysis of *p*HB-Gal as well as for that of *p*NP-Gal. IPTG also displayed similar K_i values and the same inhibition type between the enzymic hydrolyses of the two substrates. These results suggest that β-galactosyl ester and β-galactoside scramble for one active site of β-galactosidase. From these results, summarized in Table 2, it is reasonable that β-galactosidase acts on galactosyl esters and galactosides at one active site.

Kinetic behavior in the mixed substrate reaction of pHB-Gal and pNP-Gal

The implication of single active site can be supported by kinetic analysis. Hiromi *et al.* developed a kinetic theory[10] using mixed substrates to find number of active sites of a given enzyme. Their theory is useful in such a case in which released sugar is measured for determination of activity.[11] Because the procedures of galactose determination are time-consuming and a nuisance, we adopted the measurement of the aglycone for the measurement of the activity. Therefore, a method based on an other theory, explained in "Materials and Methods", was adopted instead. Mixed substrates including various concentration of *p*NP-Gal and *p*HB-Gal were hydrolyzed by β-galactosidase from *E. coli*. The hydrolytic activities for them were measured by liberation of *p*NP and *p*HBA. The ratio of hydrolytic activities for the *p*HB-Gal and *p*NP-Gal ($v_{pHB\text{-}Gal}/v_{pNP\text{-}Gal}$) were plotted (Fig. 5) against the ratios of concentrations of the *p*HB-Gal and *p*NP-Gal ([*p*HB-Gal]/[*p*NP-Gal]). As shown in Fig. 5, the most points lay along a straight line, that crossed the zero point. Ratio of $v_{pHB\text{-}Gal}/v_{pNP\text{-}Gal}$ was proportional to [*p*HB-Gal]/[*p*NP-Gal]. Therefore, the galactosyl ester and the galactoside are hydrolyzed at only one active site of β-galactosidase from the viewpoint of kinetics.

Mass spectroscopy of hydrolytic lysate containing ^{18}O

Suggestion of a single active site as above discussed brings about a problem as follows: enzymes such as lipases and esterases cleave ester linkages between carbonyl carbons and bonding oxygens. On the other hand, the fission of the linkage is between bonding oxygen and anomeric carbon in glycosidase-catalyzed reactions.[12] The galactosyl ester has anomeric carbon, bonding oxygen, and carbonyl carbon. To find which linkage in the galactosyl ester is being cleaved by β-galactosidases, hydrolysis was done using $H_2{}^{18}O$ as follows. *p*HB-Gal was hydrolyzed in 50% $H_2{}^{18}O$ by *E. coli* β-galactosidase at 40°C to form *p*HBA and galactose. The product was analyzed by FAB-MS. The spectrum is shown in Fig. 6A. Figure 6B demonstrates a spectrum of a product from reaction including no additive $H_2{}^{18}O$. The signal at M/Z 205 which is attributed to ^{18}O-galactose and sodium ion is obtained only for the hydrolysates containing $H_2{}^{18}O$. *p*HBA including ^{18}O was not detected in the spectrum for $H_2{}^{18}O$ reaction. These results demonstrated that the galactosyl ester was hydrolyzed in the same way as the galactoside was.

References

1) Nakano H., Okamoto K., Yatake T., Kiso T., and Kitahata S., Purification and characterization of a novel β-glucosidase from *Clavibacter michiganense* that hydrolyzes glucosyl ester linkage in steviol glycosides. *J. Ferment. Bioeng.*, **85**, 162–168 (1998).
2) Okamoto K., Nakano H., Yatake T., Kiso T., and Kitahata S., Purification and some properties of a β-glucosidase from *Flavobacterium johnsonae*. *Biosci. Biotechnol. Biochem.*, **64**, 333–340 (2000).
3) Nakano H., Takenishi S., and Watanabe Y., Substrate specificity of several β-galactosidases toward a series of β-1,4-linked galactooligosaccharides. *Agric. Biol. Chem.*, **51**, 2267–2269 (1987).
4) Schmidt O. T. and Reuss H., 2-[*p*-Hydroxy-benzoyl]-glucose und 2-galloyl-glucose. *Liebigs Ann. Chem.*, **649**, 137–148 (1961).
5) Takenishi S., Watanabe Y., Miwa T., and Kobayashi R., Purification and some properties of β-galactosidase from *Penicillium multicolor*. *Agric. Biol. Chem.*, **47**, 2533–2540 (1983).
6) Horikawa R. and Tanimura T., Spectrophotometric determination of carboxylic acids with 2-nitrophenylhydrazine in aquaous medium. *Proceedings of the 4th Symposium on Analytical Chemistry of Biological Components.*, 29–32 (1979).
7) Fersht A., Enzyme Structure and Mechanism. W. H. Freeman and Company, San Francisco (1977).
8) Yamasaki K., Kohda H., Kobayashi T., Kasai R., and Tanaka O., Structures of stevia diterpene-glucosides: application of ^{13}C NMR. *Tetrahedron Lett.*, **13**, 1005–1008 (1976).
9) Hiromi K., Effects of pH. In "Introduction to Kinetics of Enzymatic Reaction", ed. Ono S., Kyoritsu Shuppan, Tokyo, pp. 58–81 (1975).
10) Hiromi K., Hamauzu Z., Takahashi K., and Ono S., Kinetic studies on gluco-amylase. *J. Biochem.*, **59**, 411–418 (1966).
11) Surarit R., Matsui H., Chiba S., Svasti J., and Srisomap C., Evidence for a single active site in β-D-glucosidase/β-D-fucosidase from *Dalbergia cochinchinensis* seeds. *Biosci. Biotechnol. Biochem.*, **61**, 93–95 (1997).
12) Chiba S., Studies on molecular mechanisms in glycosidases. *Nippon Nougeikagaku Kaishi* (in Japanese), **73**, 1001–1012 (1999).