Colorimetric Assay for Evaluating Glycosyl Fluoride-hydrolyzing Activity of Glycosidase by Using Alizarin Complexon Reagent

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A novel enzyme assay for screening glycosidases has been developed by using glycosyl fluorides. The method is based on the color change caused by the complex formation of fluoride ion and lanthanum–alizarin complexon (La^{3+}–ALC). The assay showed higher sensitivity compared with the conventional method using *p*-nitrophenyl glycoside as screening substrate. A quantitative study on the hydrolysis of β-lactosyl fluoride catalyzed by a purified cellulase has been demonstrated, indicating that the present method can also be employed for enzyme kinetics.

Glycosyl fluorides are one of the most useful glycosyl donors in controlled oligo- and poly-saccharide synthesis both by chemical[1] and enzymatic[2] methodologies. The usage of glycosyl fluorides is effective particularly for the glycosidase-catalyzed glycosyl-transferring reaction in aqueous media without protection of hydroxy groups.[3] The small size and high leaving ability of fluorine atom tend to ensure reasonably accelerated reaction rates in glycosidic bond formations. Owing to the characteristics of fluorine atom, glycosyl fluorides have extensively been employed in the enzymatic glycosylation reactions catalyzed by α-amylase,[4] cellulase,[5] and glycosynthase.[6]

Finding an appropriate combination of a glycosyl fluoride and an enzyme catalyst is therefore a key to designing an enzymatic glycosylation reaction. The development of a facile procedure has been strongly required for the screening of enzymes that recognize glycosyl fluorides.[7] We postulated that if the fluoride ion liberated from a glycosyl fluoride can be detected effectively in the presence of sugar compounds, enzyme proteins, and buffer components, a new enzyme assay would be available for evaluating the glycosyl fluoride-hydrolyzing activity of glycosidases.[8]

The present paper describes a novel colorimetric assay for glycosidases based on the classical analytical method by using lanthanum–alizarin complexon (La^{3+}–ALC)[9] as coloring agent and a glycosyl fluoride as substrate. In analytical chemistry, the La^{3+}–ALC method is known to be one of the most reliable techniques to detect fluoride ion in aqueous solutions.[10] The present method consists of the glycosidase-catalyzed cleavage of the carbon–fluorine bond in a glycosyl fluoride and the subsequent complex formation of the liberated fluoride ion with La^{3+}–ALC reagent. The color change of aqueous solutions with various fluoride concentrations is shown in Figure 1A. The absorbance spectra of La^{3+}–ALC (red purple) and F^-–La^{3+}–ALC (blue purple) as well as their difference spectrum are shown in Figure 1B. The quantitative treatment of the spectral data for determination of fluoride concentration can be achieved by calculating the absorbance difference at 620 nm (dotted line in Figure 1B).[9]

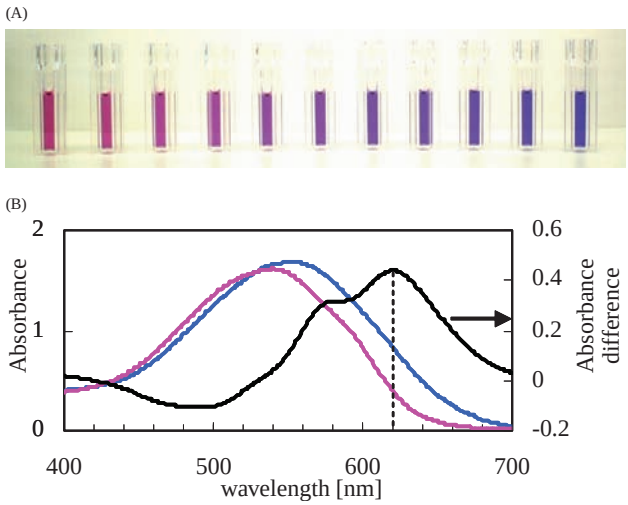
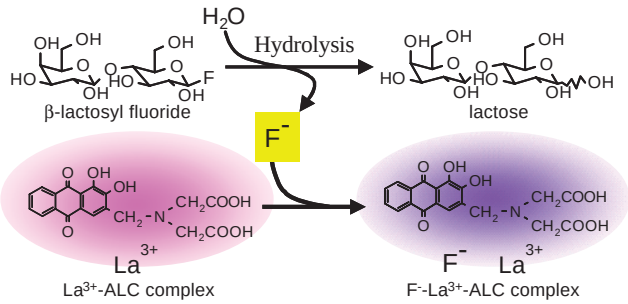


Figure 1. (A) Color changes of aqueous solutions caused by the complex formation of fluoride ion with La^{3+}–ALC. Sodium fluoride was used as fluoride ion source. The concentration of fluoride ion (μM): 9.8, 19.5, 29.3, 39.2, 48.8, 58.5, 68.3, 78.1, 87.8, 97.6, 117.1 from left to right. (B) Absorbance spectrum of La^{3+}–ALC (——) and F^-–La^{3+}–ALC (F^-: 97.6 μM) (——). Difference spectrum (——).



Scheme 1. Fluoride ion-detecting enzyme assay (FIDEA) for screening suitable enzymes by combined use of β-lactosyl fluoride and La^{3+}–ALC.

In order to confirm the reliability of the present analytical method in the presence of various chemical species, we investigated the possibility of interferences caused by sugars, proteins, metal ions, acetate, sulfate, etc. We found that these species did not interfere the colorimetric determination of fluoride ion, indicating that the complex formation is specific to fluoride ion.[11]

The present method has successfully been applied to the screening of cellulase catalysts that recognize β-lactosyl fluoride[12] (Scheme 1). Addition of the cellulase mixture caused

Table 1. Kinetic parameters for the hydrolysis of β-lactosyl fluoride substrate catalyzed by EG-III

Denaturation reagent	K_m/mM	k_{cat}/s^{-1}
50% Acetone	5.6 ± 2.0	51 ± 9
5 M Urea	4.6 ± 0.3	50 ± 1

a rapid cleavage of the anomeric carbon–fluorine bond to yield the corresponding lactose, indicating that β-lactosyl fluoride can be accepted by the catalytic site of a cellulase, liberating a fluoride ion. The hydrolysis reaction occurred via an enzyme–substrate intermediate with α-configuration (double displacement mechanism)[13] or a nucleophilic attack of water to the glycosyl oxocarbenium ion from the side opposed to that occupied by the leaving group (single displacement mechanism).[14]

On the basis of the mode of hydrolysis, cellulases are classified mostly into three categories (endo-glucanase, exo-glucanase, and β-glucosidase), and each category is composed of several glycosyl hydrolases. For the purification of an enzyme having a hydrolyzing activity from a crude enzyme preparation of Celluclast® (Novozymes Japan), five types of column chromatography were performed. Collected each fraction was assayed by the La^{3+}–ALC method, affording an active fraction that showed a single band on the SDS-PAGE. The amino acid analysis of the purified enzyme fraction clearly indicated that the sequence of the peptide fragment is identical with that of EG-III (endo-glucanase III).[15] It should be noted that EG-III enzyme can not be detected by the conventional method of using *p*-nitrophenyl glycoside derivatives as screening substrates.

In order to know whether the present method can be applicable to a kinetic study, we evaluated the hydrolysis of β-lactosyl fluoride catalyzed by EG-III quantitatively in 40 mM acetate buffer (pH 5.5). The hydrolysis was initiated by adding 7.6 mU/mL[16] of enzyme solution (6.7×10^{-9} M) at 30 °C. After appropriate intervals, the reaction mixture (25 to 100 μL) was transferred to 0.5 wt % La^{3+}–ALC solution (1 mL) containing 50 v/v% acetone or 5 M urea, resulting in the complex formation as well as termination of enzyme reaction.[17] After 90 min, the absorbance at 620 nm was observed. The kinetic parameters and their standard errors were calculated using the nonlinear regression analysis program "KaleidaGraph 3.6 J."

The kinetic parameters for the hydrolysis by EG-III were obtained from the initial rates of hydrolysis by fitting the data to the Michaelis–Menten equation (Table 1). The Michaelis constants (K_m) for β-lactosyl fluoride by EG-III in the presence of 50 v/v% acetone or 5 M urea were 5.6 and 4.6 mM, respectively. The first-order rate constants (k_{cat}) for the substrate were 51 and 50 s^{-1}, respectively.[18]

In conclusion, the fluoride ion-detecting enzyme assay (FIDEA) of glycosidases by combined use of a glycosyl fluoride and La^{3+}–ALC reagent has been developed. The present method would significantly enhance the utility of glycosyl fluorides for screening useful enzymes that catalyze a transglycosylation reaction[19] using a glycosyl fluoride as glycosyl donor in the field of glycotechnology.

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Dedicated to Professor Teruaki Mukaiyama on the occasion of his 80th birthday.

References and Notes

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16 One unit of the activity was defined as the amount of enzyme liberating 1.0 μmol of fluoride ion per minute.
17 Acetone was used to increase the sensitivity of the La^{3+}–ALC method, and urea was used to deactivate the enzyme catalyst.
18 The kinetic parameters obtained by two kinds of reactions terminated by acetone and urea are almost the same within experimental errors. The considerably large error in case of acetone may be caused by the lack of accuracy in the liquid handling.
19 The enzymatic glycosylation of several glycosyl acceptors by using β-lactosyl fluoride as donor catalyzed by EG-III leads to an efficient synthesis of the corresponding lactosides, the detail of which will be reported elsewhere as a full paper.

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