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An α-L-fucosidase from *Penicillium multicolor* as a candidate enzyme for the synthesis of α (1→3)-linked fucosyl oligosaccharides by transglycosylation

Katsumi Ajisaka *, Hiroshi Fujimoto, Mariko Miyasato

Meiji Institute of Health Science, Meiji Milk Products, Co., Ltd., 540 Naruda, Odawara 250, Japan

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Abstract

A new α-L-fucosidase was partially purified from the culture broth of *Penicillium multicolor*, which was available commercially as a freeze dried powder by the name of Lactase-P®. This enzyme catalysed the transglycosylation of fucose residue of *p*-nitrophenyl-α-L-fucopyranoside to give α-L-Fuc-(1→3)-D-Glc or α-L-Fuc-(1→3)-D-GlcNAc regioselectively. This enzyme was more stable in the organic co-solvents than the α-fucosidase from *Aspergillus niger*, which was also proposed previously by us as an enzyme to produce fucosyl oligosaccharides. © 1998 Elsevier Science Ltd. All rights reserved

Keywords: α-L-fucosidase; *Penicillium multicolor*; α-L-Fuc-(1→3)-D-Glc; α-L-Fuc-(1→3)-D-GlcNAc; Transglycosylation

1. Introduction

A variety of biologically important glycoconjugates contain α-L-Fuc residue at the non-reducing termini; for example lacto-*N*-fucopentaose I–III in human milk [1] or H, Le^x, and Le^b determinants in the blood-group substances [2]. The α-L-fucosyl residue is found with α-(1→2)-linkage to D-Gal residue or with α-(1→3)-, α(1→4)-, or α-(1→6)-linkage to D-GlcNAc residue in glycoconjugates. To elucidate the biological functions of these fucosyl oligosaccharides, many chemical syntheses have been developed [3–6]. However they were generally cumbersome due to the multiple steps of protection and deprotection processes.

On the other hand, the enzymatic method, especially the use of glycosidases, is advantageous, because of high stereo- and regioselectivity in the transglycosylation [7,8]. In the previous report [9], we have shown that the α-L-fucosidase [EC 3.2.1.5 1] from *Aspergillus niger* produced α-L-Fuc-(1→3)-Glc or α-L-Fuc-(1→3)-GlcNAc in high yield and high regioselectivity by transglycosylation using *p*NP-α-Fuc as a donor. Several biologically interesting fucosyl oligosaccharide derivatives have been synthesized with the aid of this enzyme [10,11].

* Corresponding author. Tel: 81-465-37-3661; fax: 81-465-36-2776; e-mail: rxg0120@niftyserve.or.jp

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However, the content of α-L-fucosidase activity was not satisfactory for use in a practical production of fucosyl oligosaccharides. Recently, we found a new candidate α-L-fucosidase of microbial origin exhibiting similar potentials in regioselectivity and yield in the transglycosylation. The enzyme was obtained from the culture broth of *Penicillium multicolor*, whose powdered preparation was commercially available. In the present report, we describe the characterization of this enzyme and the synthesis of α1–3 linked fucosyl disaccharides by transglycosylation.

2. Experimental

Materials.—Lactase-P™, a powdered culture broth of *P. multicolor*, was purchased from K. I. Chemical Industry Co., Ltd., (Shizuoka, Japan). *p*NP-α-Fuc was a product of Sigma. α-L-Fuc-(1→2)-Gal and α-L-Fuc-(1→6)-GlcNAc were purchased from Funakoshi Co., Ltd. (Tokyo).

Analytical Methods.—HPLC was carried out using a ÄKTA-design system (Pharmacia) with TSK G-3000 SW, Waters AccQ Tag, or Asahipak NH2P50 column. Otherwise a Dionex Bio-LC system was used with CarboPac PA-1 column and 50 mM sodium hydroxide solution as eluent. The ^{13}C NMR spectrum was recorded on a Varian Inova 500 spectrometer. Chemical shifts are expressed in ppm relative to internal acetonitrile (1.27 ppm) in D_2O.

Partial purification of α-L-fucosidase from P. multicolor.—Powder (1 g) of Lactase-P™ (α-L-fucosidase activity; 3.9 units/g) was dissolved in 10 mL of 1 mM sodium phosphate buffer (pH 6.8) and dialyzed against the same buffer. The enzyme solution was applied to Bio-Gel HTP-gel column (BioRad Labs, 2.6×11 cm), eluted with a gradient of 1 mM to 400 mM sodium phosphate buffer (pH 6.8) with a flow rate of 0.5 mL/min. Fractions (1 mL each) were collected and 10 μL of each fraction in 90 μL of 0.1 M sodium acetate buffer (pH 5.0) was incubated with 5 mM Fuc-α-*p*NP (50 μL) for 45 min at 37 °C. The absorbance at 410 nm was measured and plotted as a relative intensity of α-L-fucosidase activity. Fractions containing α-L-fucosidase activity (Fr. 28–34, 3.5 mL) were collected and concentrated to ca. 0.5 mL using Ultrafree CL™ (Millipore, molecular cut-off 30 kD). The activity of the enzyme solution was 4.4 units/mL (41.3 units/g). Here, 1 unit of α-L-fucosidase corresponds to the amount of enzyme that produces 1 μmol of *para*-nitrophenol per min. This solution was used in the following experiments without further purification.

Measurement of molecular weight.—The enzyme solution (10 μL) was applied to HPLC with TSK G-3000 SW column and eluted with 0.1 M sodium phosphate buffer (pH 7.4) containing 0.1 M sodium sulfate. When the column was eluted at a flow rate of 0.5 mL/min, the α-L-fucosidase activity was found at 15.5 min. From the calibration curve obtained by using the protein kit of Sigma, the molecular weight was estimated as ca. 180 kD.

Effect of pH and temperature.—The optimum pH was measured using 0.1 M acetate buffer and 0.1 M phosphate buffer. Enzyme solution (0.1 mL) was diluted with 1.4 mL of the buffer solution at various pHs, and the activity was measured by using *p*NP-α-Fuc. For the measurement of pH stability, 0.1 M glycine buffer, acetate buffer, and phosphate buffer were used. The enzyme solutions at various pHs were kept at 25° for 24 h. Then 0.1 mL of the enzyme solution was mixed with 1.4 mL of 0.1 M phosphate buffer (pH 5.3) and the α-L-fucosidase activity was measured.

The thermal stability was also measured by heating 0.44 unit of enzyme in 0.1 M sodium acetate buffer (pH 5.0, 1 mL) at various temperature for 30 min. Then the remaining activity was measured at 37 °C.

Effect of organic solvents.—The final concentration of the organic solvent was made to 10, 20, and 30% in 0.1 M sodium acetate buffer (pH 5.0) containing 0.1 unit of enzyme. Each solution was kept for 3 h at 37 °C, then the remaining activity was measured.

Substrate specificity.—Each disaccharide solution (0.1 mg/mL in 0.1 M acetate buffer (pH 5.0), 900 μL) was mixed with 100 μL of enzyme solution (0.01 unit), and incubated at 37 °C. An aliquot (50 μL) was withdrawn at an appropriate time intervals and filtered through a membrane of Ultrafree MC™ (Millipore, molecular cut-off 30 kD). The peak area of the remaining disaccharide in HPLC (CarboPac PA-1) was obtained by integration and plotted against time.

Synthesis of α-L-Fuc-(1→3)-D-GlcNAc by transglycosylation using partially purified enzyme.—D-GlcNAc (500 mg) and *p*NP-α-Fuc (100 mg) were dissolved in 8.5 mL of 0.1 M sodium acetate buffer (pH 5.0) containing 2 mL DMSO, then 1.5 mL of partially purified enzyme (6.2 units) was added. The

solution was incubated at 37 °C, and the reaction was monitored by HPLC with AccQ Tag column. When the peak of *p*NP-α-Fuc disappeared in HPLC the reaction was stopped by heating the solution for 5 min in a boiling water. In the case of the present experiment, the reaction was stopped at 4 h. The reaction mixture was applied onto the activated carbon column (2.7×50 cm). The column was washed with 500 mL of water and subsequently eluted with a gradient of water (1 L)–30% methanol (1 L). The sugar was detected by phenol–sulfuric acid method [12], and the fractions containing disaccharide were collected and concentrated by vacuum evaporator to give 63.5 mg of syrup. The product afforded ^{13}C NMR chemical shifts (100.8, 101.0, 8 1.6, 79.3, 62.0, 61.9, 16.7, 16.3 ppm) which supported the disaccharide to be α-L-Fuc-(1→3)-GlcNAc [9]. The yield was 49.3%.

Synthesis of α-L-Fuc-(1→3)-D-Glc by transglycosylation using crude enzyme.—D-Glc (500 mg) and *p*NP-α-Fuc (100 mg) were dissolved in 10 mL of 0.1 M sodium acetate buffer (pH 5.0) containing 10% DMF. Lactase-P® (100 mg) was added to the solution and the mixture was incubated at 37 °C. for 20 h. By the purification with activated carbon column chromatography similarly to α-L-Fuc-(1→3)-GlcNAc purification., 35.2 mg of α-L-Fuc-(1→3)Glc was isolated (27.9% yield). The structure was confirmed by comparing the chemical shifts of the characteristic peaks in ^{13}C NMR spectrum (100.4, 96.6, 93.0, 83.8, 80.8, 61.7, 61.5 ppm) with those of the literature data [9].

3. Results and discussion

For the purification of α-L-fucosidase from *P. multicolor*, Bio-Gel HTP column chromatography was performed and the result is shown in Fig. 1. Fortunately, α-L-fucosidase activity appeared at the position where the other proteins were not eluted. Therefore the activity was enhanced to 10-fold by one step purification with ca. 56% recovery.

In the examination of the effect of pH, the maximum activity was observed at pH 5 as shown in Fig. 2(a). Fig. 2(b) shows that the enzyme was stable in the pH range of 3~7. In the experiment of the thermal stability, the enzyme was stable up to 50 °C (data not shown). The thermal stability was almost the same as that of *A. niger* α-L-fucosidase. As the transglycosylation to synthesize disaccharides was generally performed at 37 °C, this enzyme was stable enough for the reaction.

As aqueous buffer is not a good solvent for Fuc-α-*p*NP, therefore the addition of organic co-solvent

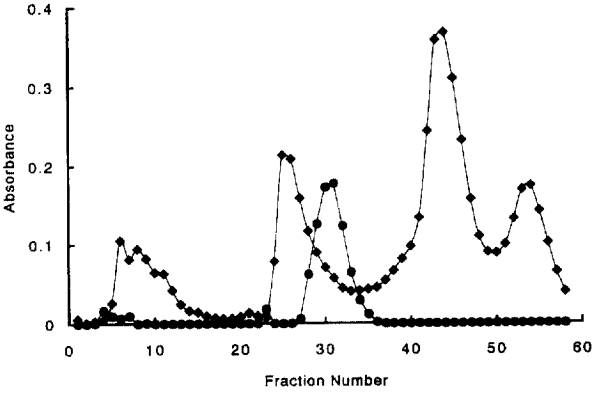

Fig. 1. Partial purification of α-L-fucosidase from crude *P. multicolor* broth by Bio-Gel HTP column chromatography. (◆); absorbances at 280 nm, (●); absorbance at 410 nm after the treatment of 10 μL of each fraction in 90 μL of 0.1 M sodium acetate buffer (pH 5.0) with 5 mM Fuc-α-*p*NP (50 μL) for 45 min at 37 °C.

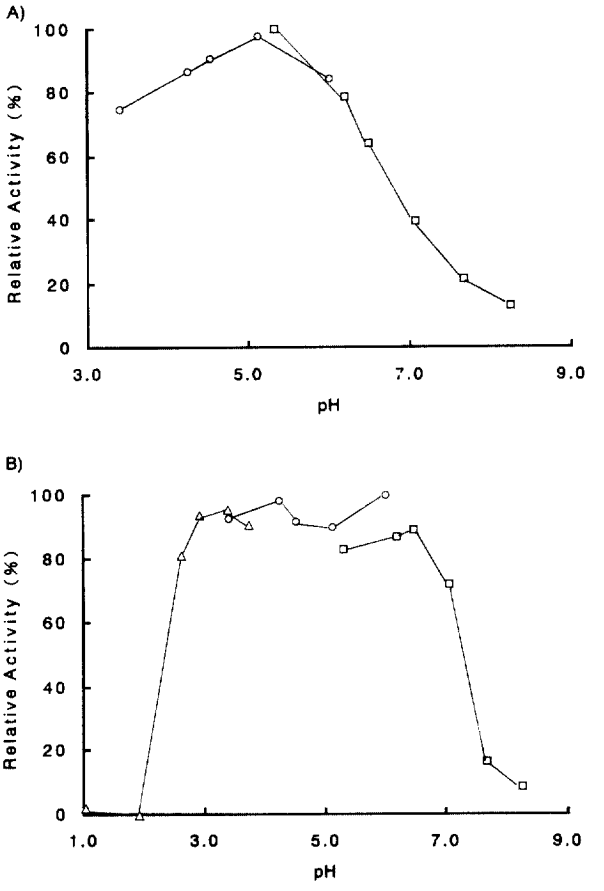

Fig. 2. Curves for optimum pH (A) and pH stability (B). (△), (○) and (□) represent the activities measured in 0.1 M glycine buffer, acetate buffer, and phosphate buffer, respectively.

Table 1
The residual α-L-fucosidase activity in various organic cosolvents

Organic solvent	Concentration (v/v%)	Residual activity %	
		A. niger	*P. multicolor*
DMSO	10%	97	100
	20%	68	100
	30%	20	58
DMF	10%	100	100
	20%	0	71
	30%	0	1
Dioxane	10%	96	100
	20%	14	55
	30%	0	0

is necessary to raise the molar ratio of donor. In that case, the stability of enzyme toward organic solvents becomes significant. Therefore, the stability of the present enzyme and α-L-fucosidase from *A. niger* was examined towards DMSO, DMF, and dioxane. Table 1 shows the relative residual activities after 3 h. The present enzyme was stable in 20% DMSO solution, while α-L-fucosidase from *A. niger* lost 33% of its activity at the same condition. For all the experiments on three organic solvents, the stability of α-L-fucosidase from *P. multicolor* was superior to that from *A. niger*.

Representative HPLC chart of the transglycosylation for the synthesis of α-L-Fuc-(1→3)-D-GlcNAc is demonstrated in Fig. 3. There is no peak corresponding to the regio-isomer around the peak of α-L-Fuc-(1→3)-D-GlcNAc (peak C) in Fig. 3, suggesting that there is no other isomer than α-L-Fuc-(1→3)-D-GlcNAc. HPLC using other column such as CarboPac PA-1 also showed only a peak of α-L-Fuc-(1→3)-D-GlcNAc in the disaccharide region (data not shown). Actually after the fractions corresponding to the disaccharide elution in the activated carbon column chromatography were corrected and concentrated to dryness, NMR was measured to give a spectrum of pure α-L-Fuc-(1→3)-D-GlcNAc.

The substrate specificity in the hydrolysis reaction was examined qualitatively at first by using α-L-Fuc-(1→2)-D-Gal, α-L-Fuc-(1→3)-D-GlcNAc, and α-L-Fuc-(1→6)-D-GlcNAc. This enzyme hydrolysed all the above disaccharides (data not shown). This result was incompatible with the result that only the α-L-Fuc-(1→3)-D-GlcNAc was synthesized by transglycosylation using GlcNAc as acceptor (Fig. 3). In this reaction, α-L-Fuc-(1→6)-D-GlcNAc should be synthesized as it was a good substrate for this enzyme, since according to our previous results [8,9] the disaccharides hydrolyzable by one glycosidase should be formed by the transglycosylation. In addition, when D-Gal was used as an acceptor, no transglycosylated product was obtained. Then we compared relative hydrolysis rate of *p*NP-α-Fuc and these disaccharides. As shown in Fig. 4, α-(1→3)-linked disaccharide was hydrolysed rather slower than α-(1→6)-isomer and α-(1→2)-isomer which were hydrolysed with a similar rate to *p*NP-α-Fuc. Therefore it can be explained that

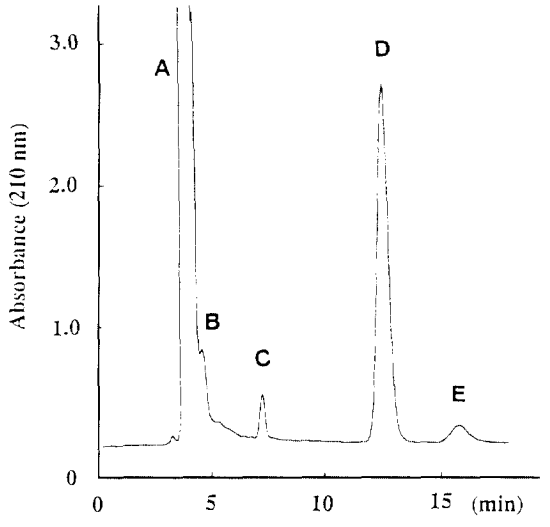

Fig. 3. HPLC of the reaction mixture after 3 h from the start of the reaction. Column:Asahipak NH2-P50 (4.6φ×250 mm); Elution: 80% acetonitrile; flow 0.8 mL/min; Detection: 210 nm. Peaks A, B, C, D, and E are assigned as solvents and *p*NP-α-Fuc, Fuc, α-L-Fuc-(1→3)-D-GlcNAc, GlcNAc, and *p*-nitrophenol, respectively.

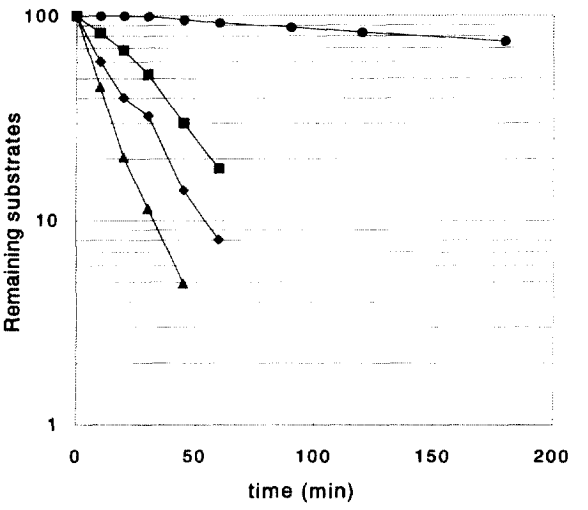

Fig. 4. Relative rate of hydrolysis of *p*NP-α-Fuc (▲), α-L-Fuc-(1→2)-D-Gal (◆), α-L-Fuc-(1→3)-D-GlcNAc (●), and α-L-Fuc-(1→6)-D-GlcNAc (■) by the present enzyme.

α-L-Fuc-(1→6)-D-GlcNAc or α-L-Fuc-(1→2)-D-Gal once formed by transglycosylation would be hydrolysed as soon as they were formed, because the formation rate of α-L-Fuc-(1→6)-D-GlcNAc or α-L-Fuc-(1→2), which was equal to the hydrolysis rate of *p*NP-α-Fuc, was almost the same as the hydrolysis rate of these disaccharides. In contrast, α-L-Fuc-(1→3)-D-GlcNAc, whose hydrolysis rate was slow, accumulated in the reaction mixture.

In the reaction using crude enzyme, the product was also unique and the reaction was also confirmed regioselective.

Although we have previously proposed the α-L-fucosidase from *A. niger* to be suitable for the production of α-(1→3)-linked fucosyl disaccharides regioselectively, the α-L-fucosidase from *P. multicolor* also exhibited a similar potential in transglycosylation reaction. The yield of α-L-Fuc-(1→3)-D-GlcNAc using the present enzyme (49%) was almost equal to *A. niger* α-L-fucosidase (58%). However, *P. multicolor* produced about five times more α-L-fucosidase than *A. niger*. Moreover, *P. multicolor* α-L-fucosidase was more stable in organic solvent than the *A. niger* α-L-fucosidase. The enhanced stability in organic solvent is not only effective in dissolving *p*NP-α-Fuc to a higher concentration, but also effective in decreasing the amount of enzyme, since the enzyme is tolerable in the extended reaction time.

In conclusion, *P. multicolor* was superior to *A. niger* in the production of α-L-fucosidase for synthesis α-(1→3)-linked fucosyl oligosaccharides.

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