Glycoconjugate Journal 18, 827–834, 2001


# An $\alpha$-L-fucosidase from *Thermus* sp. with unusually broad specificity

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**An $\alpha$-L-fucosidase (E.C. 3.2.1.51) exhibiting a wide aglycon specificity expressed in ability of cleaving $\alpha 1 \rightarrow$ 6-, $\alpha 1 \rightarrow$3-, $\alpha 1 \rightarrow$ 4-, and $\alpha 1 \rightarrow$ 2-*O*-fucosyl bonds in fucosylated oligosaccharides, has been isolated from culture filtrate of *Thermus* sp. strain Y5. The $\alpha$-L-fucosidase hydrolyzes *p*-nitrophenyl $\alpha$-L-fucopyranoside with $V_{max}$ of 12.0 $\pm$ 0.1 $\mu$M/min/mg and $K_m$= 0.20 $\pm$ 0.05 mM and is able to cleave off about 90% of total L-fucose from pronase-treated fractions of fucosyl-containing glycoproteins and about 30% from the native glycoproteins. The purified enzyme is a tetramer with a molecular mass of 240 $\pm$ 10 kDa consisting of four identical subunits with a molecular mass of 61.0 $\pm$ 0.5 kDa. The N-terminal sequence showed homology to some $\alpha$-L-fucosidases from microbial and plant sources. Hydrolysis of *p*-nitrophenyl $\alpha$-L-fucopyranoside occurs with retention of the anomeric configuration. Transglycosylating activity of the $\alpha$-L-fucosidase was demonstrated in reactions with such acceptors as alcohols, *N*-acetylglucosamine and *N*-acetylgalactosamine while no transglycosylation products were observed in the reaction with *p*-nitrophenyl $\alpha$-L-fucopyranoside. The enzyme can be classified in glycosyl hydrolase family 29.**

***Keywords:*** **$\alpha$-L-fucosidase, fucosyl-containing oligosaccharides, fucosylated glycoproteins**

***Abbreviations:*** **PNPF, *p*-nitrophenyl $\alpha$-L-fucopyranoside; Fuc$\alpha$-OMe, methyl $\alpha$-L-fucopyranoside; endo H, endo-ß-*N*-acetylglucosaminidase H from *Streptomyces griseus*.**

## Introduction

$\alpha$-L-Fucosidases (E.C. 3.2.1.51) release terminal $\alpha$-L-fucosyl residues from the carbohydrate moiety of various glycoproteins and glycolipids. Such enzymes have been found in bacteria and fungi [1,2], plants, and mammals [3–6]. $\alpha$-L-Fucosidases from various sources differ, first of all in their specificity for fucosylated oligosaccharides with different configuration of $\alpha$-L-fucosyl bonds and, secondly, in their ability to hydrolyze artificial substrates. $\alpha$-L-Fucosidases from human serum and rat tissues have broad substrate specificity and act on *p*-nitrophenyl $\alpha$-L-fucopyranoside as well as methyl $\alpha$-L-fucopyranoside [7,8]. Some microbial fucosidases, however, are able to cleave only $\alpha 1 \rightarrow$ 2-fucosyl linkages and do not catalyze the hydrolysis of artificial substrates [9,10].

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The carbohydrate moiety of glycoconjugates on mammalian cell surfaces, blood group substances, human milk oligosaccharides, serum and milk glycoproteins frequently contain $\alpha$-L-fucosyl residues at the non-reducing termini. The $\alpha$-L-fucopyranosyl group has been reported to be linked to galactopyranose residues with an $\alpha 1 \rightarrow$ 2-linkage or N-acetylglucopyranosylamine with $\alpha 1 \rightarrow$ 3-, $\alpha 1 \rightarrow$ 4- or $\alpha 1 \rightarrow$ 6 linkages [11,12]. L-Fucose accumulation, glycoprotein fucosylation, and secretion of L-fucose-containing proteins may be presumed to play numerous biological roles. Fucosyl residues of cell surface oligosaccharides, especially Fuc $\alpha 1 \rightarrow$ 3GlcNAc, was suggested to be important in cell-cell interactions [13]. Abnormal fucosylation was found to accompany diabetes [14,15], cancer [16], and certain immune diseases [17,18]. Evaluation of the $\alpha$-L-fucosidase activity in humans can become a tool for cancer diagnosis [19,20]. Presence of $\alpha$-L-fucose in N-sugar chains of human lactoferrin is possibly required for the recognition of the latter by membrane receptors of macrophages [21].

One possible approach to study the role of fucosylation is the release of L-fucose effected by specific α-L-fucosidases and α-L-fucosidases with broad specificity. In the present work enzymatic properties of the α-L-fucosidase from a selected strain of *Thermus* sp. Y5 was studied. The hydrolysis of fucosyl-containing oligosaccharides with different configuration of α-L-fucosydic linkages was analyzed, as was the reaction with the artificial substrate *p*-nitrophenyl α-L-fucoside. The ability of *Thermus* α-L-fucosidase to cleave L-fucose from N-linked sugar chains of glycoproteins was investigated with native glycoproteins and glycopeptides obtained from them.

## Materials and methods

### Chemicals

*p*-Nitrophenyl α-L-fucopyranoside (PNPF), methyl α-L-fucopyranoside (Fucα-OMe), *N*-acetylglucosamine (GlcNAc), *N*-acetylgalactosamine (GalNAc), *p*-nitrophenyl α-D-fucopyranoside and other *p*-nitrophenyl α/β-glycosides were purchased from Sigma, St. Louis, USA, L-[$^3$H]fucose (23 Ci/mM) was from "Isotope", Russia. Oligosaccharide **1** was synthesized according to the procedures described in [22], **2–5** indicated below were obtained according to the method described in [23], 6-α-Fucosyl chitobiose (**6**) was purchased from Sigma.

**1** α-L-Fuc-(1 → 2) − β-D-Gal-(1 → 3)-D-GalNAc
**2** α-L-Fuc-(1 → 2)-β-D-Gal-(1 → 4)-β-D-GlcNAc-1 → Me
**3** α-L-Fuc − (1 → 3)-β-D-GlcNAc-1 → OMe
**4** α-L-Fuc-(1 → 4)-β-D-GlcNAc-1 → OMe
**5** α-L-Fuc-(1 → 2)-β-D-Gal-1 → OMe
**6** β-D-GlcNAc-(1 → 4)-[α-L-Fuc-(1 → 6)]-D-GlcNAc

L-Fucose dehydrogenase (6-deoxy-L-galactose NADH 1-oxidase) from pork liver was purified essentially as reported by Schachter, H. et al. [24] with an additional purification step on a DEAE 5PW column (21.5 × 150 mm) from Pharmacia, LKB. Fractions containing L-fucose dehydrogenase were transferred to 5 mM sodium phosphate buffer, pH 7.4 and applied onto the column equilibrated with the same buffer. The enzyme was eluted with a linear gradient of 0 to 0.5 M NaCl (total volume 160 ml) in 5 mM phosphate buffer, pH 7.4 at a flow rate of 2.5 ml/min. Purified fucose dehydrogenase contained less than 0.001 U per mg of α-L-fucosidase activity.

### Isolation of the strain and microorganism growth

Thermophilic strain *Thermus* sp. Y5 was isolated from soil samples collected from thermal sources at Kamtchatka. Cultures with α-L-fucosidase activity were selected on agar plates containing *p*-nitrophenyl α-L-fucopyranoside as an indicator. After incubation at 48°C for 12 h followed by flooding with 1 M $Na_2CO_3$ the *Thermus* strain Y5 gave clear yellow zones. The fucosidase-producing isolate was finally purified by subculturing three times on LB (Luria-Bertani) plates. To produce α-L-fucosidase in amounts sufficient for biochemical analysis, the culture was inoculated into a 2-liter flask with 500 ml of a liquid medium consisting of 3% suspension of disrupted cell walls of *Saccharomyces cerevisiae* and mineral salts, g/l: $Na_2HPO_4$, 6; $KH_2PO_4$, 3; NaCl, 0.5; $NH_4Cl$, 1. After 16-h cultivation with shaking at 45°C, the culture broth was transferred to a 20-liter fermenter containing 5 l of the same medium. Cultivation was continued for 2 days at 45°C with aeration (2 l/min) and stirring (700 rpm).

### Analytical methods

Molecular mass of the protein was estimated by SDS-PAGE on 10% polyacrylamide gels according to Laemmli [25] using a molecular weight calibration kit LMW (14,400–94,000) from Pharmacia, Sweden. Determination of the apparent molecular mass of the native protein in non-denaturing conditions was performed by analytical chromatography on a Superose 12 (Pharmacia) column (10 × 300 mm) equilibrated with 20 mM sodium phosphate, pH 6.5, 100 mM NaCl. Kit (MW-GF-200) for molecular weights 12,000–200,000 from Sigma was used for calibration of the column. Isoelectric focusing was on Servalyt PRECOATES plates 3–10, "Serva", Germany. Protein concentration was measured following the Lowry procedure using BSA as a standard [26]. The N-terminal sequence of the purified protein was determined by using a Procise 494A sequencer (Perkin-Elmer, Applied Biosystems Division, Foster City, CA, USA). Radioactivity was measured using liquid scintillation counter BETAMAN 1206 (from Pharmacia, Germany). Purity of the substrates was evaluated by NMR and TLC methods. $^1$H NMR spectra were recorded with an AMX-500 Bruker spectrometer. TLC was on Kieselgel 60 plates (Merck) with a mobile phase of butan-1-ol/acetic acid/water (3:2:1, by vol.). For evaluation of intracellular α-L-fucosidase activity, cells were incubated with 0.3 % lyzozyme (w/v) at 20°C for 60 min, lyzed by sonication (3 × 20 sec), and centrifuged (12000 × g, 20 min). The resulting supernatant was used for enzyme assays.

### Purification of the α-L-fucosidase

Unless otherwise stated all purification steps were at 4°C in 20 mM Tris HCl buffer, pH 7.2, supplemented with 3 mM DTT (buffer A). The culture supernatant was concentrated 10-fold using hollow fibers ("Kirishi", Kirishi, Russia) and transferred to buffer A before application onto a DEAE Toypearl column (15 × 120 mm). Bound proteins were eluted with 1 M NaCl (total volume 200 ml) at a flow rate of 1.0 ml/min. Fractions with α-L-fucosidase activity were pooled and dialyzed against buffer A and loaded onto a DEAE 5PW column (21.5 × 150 mm) equilibrated with the same buffer. Elution was with a linear gradient (0–1 M) of NaCl in buffer A (total volume 160 ml, flow rate 2.0 ml/min). Fractions containing α-L-fucosidase activity were pooled, desalted by dialysis against buffer A and chromatographed on a Mono Q HR5/5 column

(Pharmacia Biotech) equilibrated with buffer A. Elution was with a linear gradient (0–1M) NaCl (total volume 24 ml) at a flow rate of 0.6 ml/min. Then the enzyme was further purified using an L-fucosylamine-agarose affinity column (Sigma, Cat. No. F3902) equilibrated with buffer A. Enzyme fractions were eluted with 40 mM L-fucose and dialyzed against buffer A. Finally, the α-L-fucosidase was solved in 1.7 M $(NH_4)_2SO_4$and applied onto a Phenyl Superose HR 5/5 (Pharmacia) column equilibrated with 1.7 M $(NH_4)_2SO_4$ in 20 mM Tris HCl buffer, pH 7.2. The column was washed with 20 mM Tris HCl buffer and the bound protein was eluted with a decreasing concentration of ammonium sulfate (1.7–0 M) in Tris HCl, pH 7.2 (total volume 24 ml) at a flow rate of 0.6 ml/min. Purified enzyme was dialyzed against deionized water with 3 mM DTT followed by addition of 20 mg/ml of bovine serum albumin (BSA) to the desalted protein solution and lyophilization.

### Enzyme assays and enzymatic properties

Enzyme activity was measured at 37°C in 50 mM sodium phosphate buffer, pH 6.5, using PNPF as a substrate in accordance with [27]. One unit of α-L-fucosidase activity towards PNPF was defined as the amount of enzyme required to hydrolyze 1 μM of substrate per min at 37°C in 50 mM sodium phosphate buffer, pH 6.5. The enzyme activity in hydrolysis of other *p*-nitrophenyl α/β-glycosides was measured in the same manner. Activity of the α-L-fucosidase in the hydrolysis of fucosylated oligosaccharides and Fucα-OMe was assayed by L-fucose liberation using $NAD^+$-dependent fucose dehydrogenase according to the procedure described by Tsay et al. [28].

The influence of pH and temperature on activity of the enzyme was studied with PNPF as a substrate. The effect of pH on activity was measured at 37°C for 10 min in the range pH 3–pH 9 in 100 mM sodium phosphate-citrate buffers. Reaction mixtures contained 200 μl of buffer, 10 μl of the enzyme (0.01 U) solution in 5 mM Tris HCl buffer, pH 7.2, and 10 μl of 10 mM PNPF. The influence of pH on stability of the enzyme was studied by incubating enzyme samples at 30°C for 16 h in 100 mM sodium phosphate-citrate buffers ranging from pH 3 to pH 9, followed by measuring the residual activity using standard conditions. The effect of temperature on activity was measured at pH 6.5 in 50 mM PIPES buffer over a temperature range of 25–80°C. The reaction mixture (0.2 ml) containing 5 mM PNPF was pre-incubated at various temperatures in the range mentioned above. Then 10 μl (0.005 U) of enzyme solution in the same buffer was added and the activity was measured using standard conditions. Temperature stability of the α-L-fucosidase was examined after treating aliquots of enzyme (0.02 U) at various temperatures for 10 min followed by cooling to 37°C and determination of activity using standard conditions.

Effect of metal ions on α-L-fucosidase activity towards PNPF was measured in 50 mM PIPES buffer, pH 6.5, at 37°C after 5 min of incubation with 2 mM of different ions.

Values for Michaelis constants were calculated for PNPF, Fucα-OMe, and fucosylated oligosaccharides **1**,**2**, and **6** from the Michaelis-Menten equation by non-linear regression analysis [29]. The hydrolysis was in 50 mM sodium phosphate buffer, pH 6.5, at 37°C at concentrations of PNPF ranging from 1.5 to 0.05 mM, Fucα-OMe in the range from 30 to 0.1 mM, and compounds **1**, **2**, and **6** from 20 to 0.2 mM. The kinetic parameters were defined from the Lineweaver-Burk plot and inhibition constant for L-fucose was measured by the Dixon method varying the substrate concentration in the reaction mixture [30]. The substrate specificity of the α-L-fucosidase was studied by analyzing α-L-fucosidase-catalyzed liberation of L-fucose from fucosylated oligosaccharides **1**–**6** for different time intervals at 37°C in 50 mM sodium phosphate buffer, pH 6.0.

### NMR investigations

All NMR measurements were performed at ambient temperature (≈290 K). Prior to NMR analysis the purified α-L-fucosidase, substrate (PNPF) and buffer materials were freeze-dried twice from $D_2O$. The measurements were made in 20 mM sodium phosphate buffer (pD 6.5) at room temperature. The enzyme was dissolved in 30 mM sodium phosphate buffer, pH 6.5, made up in $D_2O$. The reaction mixture of 0.6 ml contained 3.1 mg of PNPF and 3 U of the α-L-fucosidase. Concentrations of PNPF and α- and β-L-fucose were determined by integration of respective signals of C-6 methyl groups at $\delta = 1.163$, 1.206 and 1.247 ppm for PNPF, α- and β-anomers of L-fucose, respectively. The reaction was monitored by collecting $^1H$ NMR spectra once per minute after enzyme addition. Anomeric proton signals (α- and β-) were registered during the course of the reaction.

### Analysis of transglycosylating activity

All experiments on transglycosylating activity of the α-L-fucosidase were carried out in 50 mM sodium acetate buffer, pH 6.5, 37°C using 0.1–0.2 U/ml of enzyme for different time intervals. The reactions were terminated by freezing and lyophilzation. Reaction of substrate transglycosylation with PNPF was investigated at substrate concentrations ranging from 5 mM up to 18 mM. Reaction mixtures were analyzed by TLC as described above and by HPLC using the Waters Spherisorb C8 column (200 × 4.8 mm) according to [31]. Analysis of products of substrate transglycosylation with Fucα-OMe as substrate was performed on a Lichrosorb $NH_2$ column (Pharmacia, LKB) according to the procedures described in [32]. To examine the transglycosylation activity of the α-L-fucosidase towards alcohols as acceptors, the reactions were with 10 mM PNPF as a fucosyl donor. Acceptor concentrations were in the range of 5–15% (v/v). Transglycosylation activity of the enzyme with GalNAc and GlcNAc as acceptors and 10 mM Fucα-OMe as a donor was investigated at acceptor concentrations in the range of 10–50 mM. The formation of transglycosylation products was analyzed by TLC and by HPLC on a Lichrosorb-$NH_2$ column [32]. Moreover, after product separation by HPLC, GLC-MS

**Table 1.** Purification of the $\alpha$-L-fucosidase

| *Purification step* | *Volume* | *Total protein* | *Total activity* | *Specific activity* | *Purification* | *Yield* |
|---|---|---|---|---|---|---|
| | ml | mg | U | U/mg | -fold | % |
| Cultural liquid | 5000 | 250 | 60 | 0.24 | 1 | 100 |
| Ultrafiltration | 200 | 175 | 55 | 0.30 | 1.3 | 90 |
| DEAE-Sephadex chromatography | 50 | 70 | 35 | 0.50 | 2.0 | 50 |
| DEAE-5PW chromatography | 25 | 30 | 25 | 0.83 | 3.5 | 42 |
| Affinity chromatography | 2 | 2.1 | 20 | 9.6 | 40 | 33 |
| Phenyl Superose chromatography | 3 | 1.8 | 18 | 10 | 41.7 | 30 |

and $^1$H NMR methods were used for their identification as described in [31,32]. Calculation of the yield of transglycosylation products was done according to the methods reported by Vetere et al. [33].

### Preparation of L-[$^3$H]fucose-labeled glycoproteins and glycopeptides

Permanent cell line of Chinese Hamster fibroplasts (V79) was provided by Institute of Cytology, RAS, St. Petersburg, Russia, and grown in Eagles minimum essential medium containing 10% of BSA [34]. Radioactive labeling was accomplished by incubating cells with 200 $\mu$Ci of L-[$^3$H]fucose (23 Ci/mM) on 100-mm dishes (Falcon) for 12 h. At the end of cell incubation, the medium was carefully removed, and the plates were washed twice with 2 ml of phosphate-buffered saline. Cell lysates were prepared according to the procedures reported by Pählsson and Spitalnik [35]. Total glycoprotein fraction was obtained after subsequent centrifugation at 12,000 *g* and chromatography on a DEAE-Toypearl column in accordance with [36]; this fraction was used in further investigations. Glycopeptides from the total fraction of L-[$^3$H]fucose-labeled glycoproteins were obtained by pronase digestion [36]. N-sugar chains were released by endo-$\beta$-N-acetylglucosaminidase F from *Flavobacterium meningosepticum* (Boehringer Mannheim GmBH, Germany) according to [37]. For quantification of radiololabeled fucose samples were subjected acid hydrolysis [38] followed by passage through coupled columns of Dowex 50 ($H^+$ form) and Dowex 1 (acetate form). Fucose was isolated by TLC in ethanol/butan-1-ol/$H_2O$ (2:2:1, by vol.); radioactivity was determined by scintillation counting after elution of the component from the plate. Radiolabeled glycoproteins and glycopeptides were treated with $\alpha$-L-fucosidase at 37°C, 20 mM sodium phosphate buffer, pH 6.5, for different intervals of time. L-[$^3$H]Fucose released by the $\alpha$-L-fucosidase was determined by scintillation counting as described above.

## Results

Using four chromatographic steps the $\alpha$-L-fucosidase was purified approx. 42-fold from concentrated cultural supernatant of *Thermus* sp. with a yield of 30% of the initial activity. The purified enzyme preparation had a specific activity of 10 U·mg$^{-1}$ (Table 1). SDS-PAGE analysis revealed a single polypeptide with an apparent molecular mass of 61.0 ± 0.5 kDa (Figure 1). Analytical gel-filtration on a Superose 12 column showed that the native $\alpha$-L-fucosidase had an apparent molecular mass of 240 ± 10 kDa. Since only a single N-terminal amino acid sequence was obtained the values for molecular mass determined under denaturing and non-denaturing conditions suggest that the enzyme is composed of four identical subunits. The $\alpha$-L-fucosidase was found to be a secreted protein. The total $\alpha$-L-fucosidase activity was distributed as 12:1 between the supernatant and cell fraction. The intracellular activity was measured after cell disruption as described in Materials and methods. During the purification process glycosidase activities initially present in the cultural liquid ($\beta$-1→3-glucanase, $\beta$-glucosidase, $\alpha$-mannosidase, $\beta$-mannosidase, $\beta$-xylosidase and others) were efficiently removed resulting in less than 0.01% of contaminant activity. Pure *Thermus* $\alpha$-L-fucosidase did not hydrolyze *p*-nitrophenyl $\beta$-D-fucoside and *p*-nitrophenyl $\beta$-L-fucopyranoside, *p*-nitrophenyl N-acetyl-$\beta$-D-glucosaminide, and *p*-nitrophenyl $\alpha$-mannopyranoside. High purity of the enzyme was achieved in four steps using affinity chromatography on an amino-fucosyl-L-Sepharose column as an effective tool for removing minor contaminating glycosidase activities. The purified $\alpha$-L-fucosidase displayed optimal activity in the hydrolysis of PNPF at pH 6.5. The optimal temperature for hydrolysis was 50°C. However, thermal inactivation was observed to occur at temperatures above 45°C. Activity decreased rapidly at temperatures above 60°C. The enzyme was stable overnight in the pH range from 5.5 to 7.5 at 37°C. Pure preparations of $\alpha$-L-fucosidase freeze dried from an aqueous solution containing 3 mM DTT and BSA (5–20 mg/ml) stored at 4°C retained full activity for at least one year. The isoelectric point was 4.2 ± 0.05. The N-terminal amino acid sequence: MNPRFERTLPLMNDRFGLFIHWGLPAIPAR was compared to sequences of the proteins by Basic Local Alignment Search Tool (BLAST) on the web site of the National Center for Biotechnology Information, http://www.ncbi.nlm.nih.gov:80/BLAST/) [39]. The highest homology for the short fragment (Score = 28, E value = 18) was found with $\alpha$-L-fucosidase from *Xanthomonas campestris*

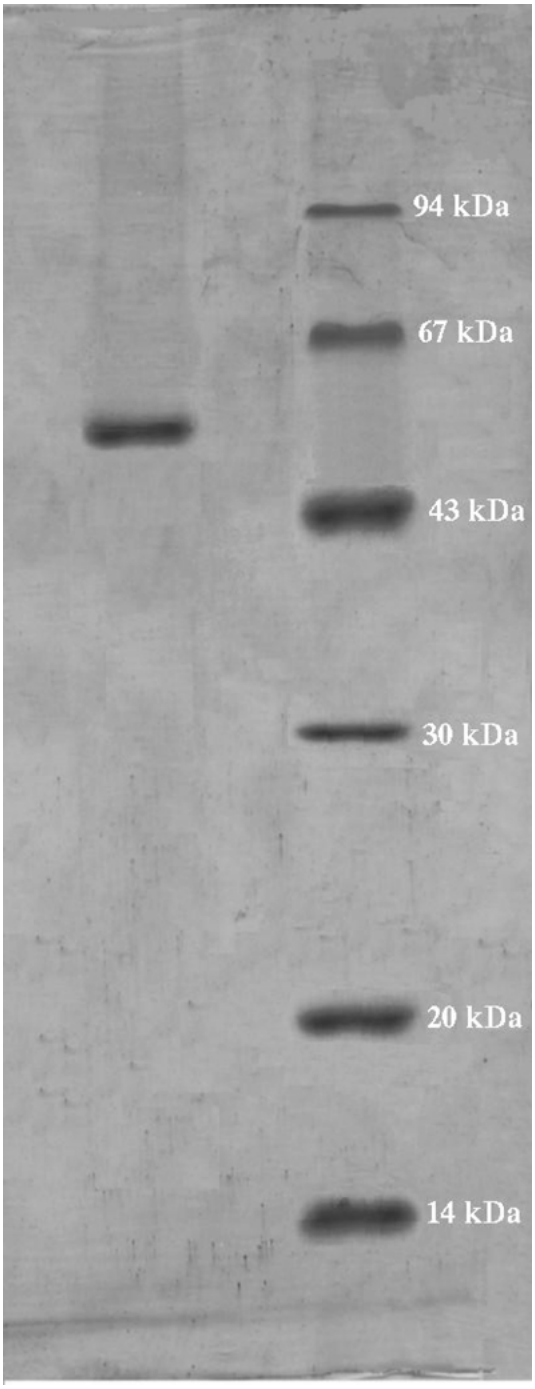


**Figure 1.** SDS-PAGE of the α-L-fucosidase from *Thermus* sp. Lane 1, purified α-L-fucosidase from *Thermus* sp.; lane 2, protein standards—phosphorylase b from rabbit (94 kDa), BSA (67 kDa), egg white ovalbumin (45 kDa), bovine erythrocyte carbonic anhydrase (30 kDa) soybean tripsin inhibitor (20.1 kDa) bovine milk α-lactalbumin (14.4 kDa).

(gi:21229478). A highly conserved region in sequences of α-L-fucosidases is shown in Figure 2. The region has been identified as a pfam01120.4 domain of α-L-fucosidases (accession number of PFAM:PFO1120) [40].

Treatment with EDTA did not affect the α-L-fucosidase activity. Divalent and trivalent metal ions had only a little influence on the enzyme activity except for PHMB, and $Hg^{2+}$, which completely destroyed the activity. Neither cystamine nor β-mercaptoethanol restored the α-L-fucosidase activity after mercury compound treatment.

*Thermus* α-L-fucosidase hydrolyzed all α-L-fucosyl linkages in fucosyl-containing oligosaccharides investigated. The most common types of α-fucosidic linkages found in different glycoconjugates including Fuc α1→2Gal (compounds **1**, **2**, and **5**) and Fuc α1→3-, α1→4-, α1→6-GlcNAc (compounds **3**, **4**, **6**) were used as model substrates (Tables 2 and 3). It is noteworthy that all initial velocities of the hydrolysis are within the same order of magnitude. α-L-Fucosidase was capable of releasing terminal L-fucose from short linear fucosylated oligosaccharides with DP >2 (**1**, **3**–**5**) and from fucosyl-containing branched structures (**2** and **6**). Values for $K_m$ and $V_{max}$ in the hydrolysis of **1**, **2**, and **6**, calculated from Lineweaver-Burk plots, were quite similar (Table 2). With PNPF the value for $V_{max}$ was estimated to be about 10-times higher than for other fucosylated oligosaccharides. As expected, Fucα-OMe was also substrate for the α-L-fucosidase. Kinetic parameters for its hydrolysis were calculated and shown in Table 2. It should be noted that PNPF was the best substrate for the α-L-fucosidase. α-L-Fucose was found to be a competitive inhibitor of hydrolysis of PNPF with an inhibition constant $K_I$ of 0.150 ± 0.005 mM.

The stereochemical course of hydrolysis catalyzed by the α-L-fucosidase from *Thermus* sp. was investigated by $^1$H NMR

**Table 2.** Kinetic parameters of reactions catalyzed by α-L-fucosidase

| *Substrate* | $K_M$ *(mM)* | $V_{max} \times 10^3$ *(mM/min × mg)* |
|---|---|---|
| PNPF | 0.2 | 12 |
| Fuc-αOMe | 16 | 0.152 |
| **1**: α-L-Fuc-(1→2)-β-D-Gal-(1→3)-D-GalNAc | 3 | 0.142 |
| **2**: α-L-Fuc-(1→2)-β-D-Gal-(1→4)-β-D-GlcNac-1→Me | 7 | 0.38 |
| **6**:β-D-GlcNAc-(1→4)-[α-L-Fuc-(1→6)]-D-GlcNAc | 9 | 0.227 |

**Table 3.** Initial rates of fucose liberation from various fucooligosaccharides catalyzed by the α-L-fucosidase

| *Substrate* | $V_0 \times 10^3$ *(mM/min × mg)* |
|---|---|
| **3**: α-L-Fuc-(1→3)-β-D-GlcNAc-1→OMe | 0.933 |
| **4**: α-L-Fuc-(1→4)-β-D-GlcNAc-1→OMe | 0.52 |
| **5**: α-L-Fuc-(1→2)-β-D-Gal-1→OMe | 0.963 |
| **6**: β-D-GlcNAc-(1→4)-[α-L-Fuc-(1→6)]-D-GlcNAc | 0.12 |

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Thermus sp.                MNPRF--------ERTLPLMND--RFGLFIHWGLPAIP-
Xanthomonas campestris    -MKPRYKPDWESLREHTVPKWFDKAKFGIFIHWGIYSVP-
Xanthomonas axonopodis    -APHHYTPDWPSLDSRPLPRWFDEAKFGLFVHWGVYSVP-
Thermotoga maritima       -PPRRYTPDWQSLDSRPLPDWFDKAKFGVFVHWGVFAVP-
Xylella fastidiosa        -RKGPFRPDWAALKAYQSPAWYDNAKFGIFIHWGVFSVP-
rat                       -RKGPFRPDWAALKAYQSPAWYDNAKFGIFIHWGVFSVP-
Canis familiaris          -RKGPFRPDWAALKAYQSPAWYDNAKFGIFIHWGVFSVP-
Sulfolobus solfataricus   --NGPFKPTWSSLKKYIVPSWFTTSKFGIFIHWGVYSVP-
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**Figure 2.** Conserved region in the sequences of $\alpha$-L-fucosidases. Conserved sequences are aligned with N-terminal sequence of the *Thermus* $\alpha$-L-fucosidase. Identical and similar amino acid residues are enclosed in black boxes and gray boxes, respectively.

spectroscopy. The time course of accumulation of the $\alpha$- and $\beta$-anomers of L-fucose resulting from hydrolysis of PNPF is presented in Figure 3. From these data, the $\alpha$-anomer of L-fucose is formed during the initial stage of the hydrolysis. At later stages the $\beta$-anomer is accumulating as a result of mutarotation.

The $\alpha$-L-fucosidase did not reveal any transglycosylating activity the reaction with PNPF and Fuc$\alpha$-OMe, at least in the range of the reagents concentrations (up to 18 and 50 mM, respectively). However, when methanol or ethanol was used as acceptors, Fuc$\alpha$-OMe and ethyl $\alpha$-L-fucoside were formed (determined by $^1$H NMR spectroscopy). At a concentration of 15% (v/v) of methanol or ethanol maximal yields of alkyl fucopyranosides (40 and 35%, respectively) were achieved. L-fucopyranosyl 2-acetamido-2-deoxyl-D-glucopyranoside and L-fucopyranosyl 2-acetamido-2-deoxyl-D-glucopyranoside were formed with a yield of 20% at an acceptor concentration of 50 mM. The configuration of $\alpha$-fucosyl linkages in mentioned products was not determined in this work.

The ability of $\alpha$-L-fucosidase to cleave off L-fucose from N-linked sugar chains of native glycoproteins and pronase-generated glycopeptides was checked using the total fraction of L-[$^3$H]fucose-labeled material obtained from a cell line of Chinese hamster fibroplasts. It was found , these glycoproteins and glycopeptides contain mono- and bi- and tetraantennary complex-type N-sugar chains which linked by Fuc $\alpha 1 \rightarrow 6$ bond at the Asn-bound *N*-acetylglucosamine. Possibly, in bifucosylated N-linked sugars one fucose residue is $\alpha 1 \rightarrow 3$-linked with the peripheral GlcNAc residue [41–43]. Independent experiments demonstrated that up to $95 \pm 3\%$ of L-[$^3$H]fucose was released from pronase-digested fractions by Endo F treatment leading to conclusion that we were dealing with L-[$^3$H]fucose incorporated into N-linked sugar chains of glycoproteins. *Thermus* $\alpha$-L-fucosidase cleaved 30% of total $\alpha$-L-fucose from glycoproteins (24 h, 2 U of $\alpha$-L-fucosidase per 1 mg of the substrate). Increasing the time of treatment or the concentration $\alpha$-L-fucosidase did not lead to complete release of L-fucose from glycoprotein whereas action of the same quantity of the $\alpha$-L-fucosidase on glycopeptides gave $25 \pm 3\%$ release of fucose after the first 2 h of incubation and $90 \pm 3\%$ after 24 h.

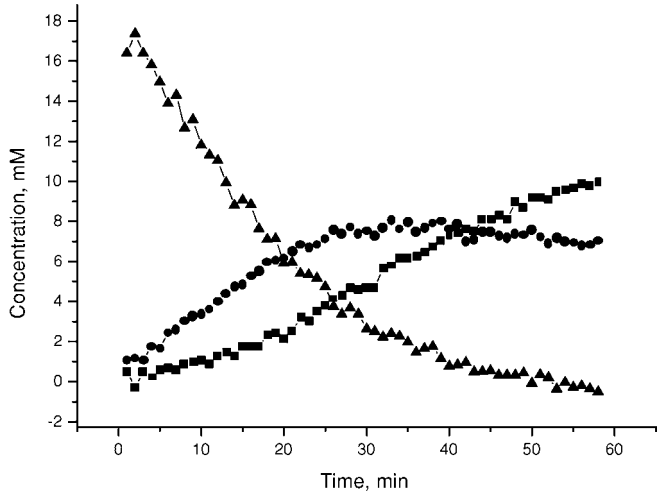


**Figure 3.** Kinetics of $\alpha$- and $\beta$-fucose formation during the hydrolysis of PNPF. ●, $\alpha$-fucose; ■, ß-fucose; ▲, PNPF. The reaction was made in accordance with conditions described in Materials and methods.

## Discussion

The N-terminal sequence of an $\alpha$-L-fucosidase isolated from *Thermus* sp. displayed sequence similarities with $\alpha$-L-fucosidases from various sources. The stereochemical course of the glycanase-catalyzed hydrolysis was readily analyzed by $^1$H NMR spectroscopy and integration of relevant signals for C-6 methyl group protons in *p*-nitrophenyl $\alpha$-fucoside and $\alpha$- and $\beta$-anomers of L-fucose with the distinct shifts at $\delta = 1.163$, 1.206 and 1.247 ppm, respectively. The data obtained for *Thermus* $\alpha$-L-fucosidase demonstrate that hydrolysis proceeds with retention of the anomeric configuration, presumably as a result of a single displacement reaction [44]. The $\alpha$-L-fucosidase can be classified in glycosyl hydrolase family 29 according to Henrissat [45]. Transglycosylating ability of the *Thermus* $\alpha$-L-fucosidase , particularly in enzymatic syntheses of fucosyl-containing oligosaccharides and alkyl fucosides is of potential interest for biotechnology and experiments are in progress. Transglycosylation activity has been demonstrated for the $\alpha$-L-fucosidases from *Aspergillus niger* [33] and *Penicillium multicolor* [46]. Although no direct stereochemical experiments have been reported for these enzymes, it may be assumed that they catalyze hydrolysis with retention of the anomeric configuration. Both fucosidases have been used in regioselective

synthesis of biologically important fucosyl-containing oligosaccharides including Fuc α1→3Glc and Fuc α1→3GlcNAc.

The α-L-fucosidase from *Thermus* sp. display similar kinetic characteristics in hydrolysis of PNPF as human and other mammalian α-L-fucosidases. Indeed, human liver fucosidase hydrolyzes this substrate with apparent values for $K_m$ of 0.43 mM and $V_{max}$ of 19.6 μM/(min·μg) [27]. Kinetic parameters for rat liver lysosomal enzyme were 0.19 mM and 27 μM/(min·μg), respectively [47]. α-L-Fucose was a competitive inhibitor in PNPF hydrolysis for both fucosidases similarly to the considered α-L-fucosidase. Nevertheless, due to the specificity of *Thermus* α-L-fucosidase towards L-fucosyl residues attached to carbohydrate moieties of glycoproteins, it differs from previously isolated microbial fucosidases, which are capable of hydrolyzing fucosyl-containing oligosaccharides with a limited number of α-fucosyl linkage configurations. For example, α-L-fucosidase from *Aspergillus orizae* was reported to hydrolyze only Fuc α1→2Gal pyranosyl linkages [10] and *Streptomyces* sp. enzyme was able to release L-fucose from α1→6-fucooligosaccharides [1]. Data obtained for the α-L-fucosidase described here showed that the enzyme cleaved terminal L-fucosidic bonds in fucosyl-containing oligosaccharides with α1→2-, α1→3-, α1→4, and α1→6-configuration, displaying an extensive aglycon specificity. Compared to rat liver [47] and rat epididymis [48] α-L-fucosidases with similar property, *Thermus* α-L-fucosidase was observed to hydrolyze L-fucosidic linkages from not only carbohydrate fragments of glycopeptides but also of carbohydrate moieties of glycoproteins. An important feature of the considered α-L-fucosidase is its ability to cleave practically all L-fucose (up to 90%) from N-linked sugar chains of a glycoprotein fraction in contrast to a corresponding enzyme from *Bacillus circulans* which is capable of hydrolyzing only α1→6-fucosidic bonds in fucosylated oligosaccharides [1]. The maximal *Thermus* α-L-fucosidase catalyzed release of fucose from glycopeptides during the first 2 h of incubation did not exceed 25%, irrespective of enzyme concentration. Extending the time of incubation led to liberation of the remaining fucose, but at a lower rate. This observation may be explained by the presence of various branched structures of N-sugar-containing glycopeptides or by different velocities of the hydrolysis of mono- and bifucosylated N-sugar chains (Table 3) [48]. Being microbial enzyme, *Thermus* α-L-fucosidase with broad specificity can be produced in large quantities and are therefore better suited for biotechnological purposes than mammalian fucosidases with similar properties. Due to its specific hydrolytic ability, *Thermus* α-L-fucosidase can be an effective tool for the production of deglycosylated forms of fucose-containing glycoproteins with various types of linkage configuration.

## Acknowledgments

This work was supported in part by the grant of 00-04-48878 from the Russian Foundation for Basic Research and the grant of 6-th Expertise-Competition from the Presidium of Russian Academy of Science. We thank Dr. Filatov M.V., PNPI, RAS, for his essential assistance with fibroplasts preparation and Prof. Karl K. Thomsen for his reviewing the manuscript and valuable comments.

## References

1 Tsuiji Y, Yamamoto K, Tochikura T, Purification and some properties of a novel α-L-fucosidase capable of acting on α-(1→6)-L-fucosidic linkages from *Bacillus circulans* M28, *J Biochem* **108**, 235–40 (1990).
2 Sano M, Hayakawa K, Kato I, Purification and characterization of α-L-fucosidase from *Streptomyces* species, *J Biol Chem* **267**, 1522–7 (1992).
3 Scudder P, Neville DCA, Butters TD, Fleet GWJ, Dwek RA, Rademacher TW, Jacob GS, The isolation by ligand affinity chromatography of a novel form of α-L-fucosidase from almond, *J Biol Chem* **265**, 16472–7 (1990).
4 Codina A, Vilaseca M, Tarrago T, Fernandez I, Ludevid D, Giralt E, Location of disulfide bonds in mature α-L-fucosidase from pea, *J Pept Sci* **7**, 305–15 (2001).
5 Abascal I, Skalaban SR, Grimm KM, Aviles M, Martianez-Menarg JA, Castells MT, Ballesta J, Alhadeff JA, Alteration of the isoform composition of plasma-membrane-associated rat sperm α-L-fucosilate epididymal maturation: Comparative characterization acidic and neutral isoforms, *Biochem J* **333**, 201–7 (1998).
6 Alhadeff JA, Khunsook S, Choowongkomon K, Baney T, Heredia VA, Bean B, Characterization of human semen α-L-fucosidases, *Mol Hum Reprod* **5**, 809–15 (1999).
7 DiCioccio RA, Barlow JJ, Matta KL, Substrate specificity and other properties of alpha-L-fucosidase from human serum, *J Biol Chem* **257**, 714–8 (1982).
8 Aviles M, Abascal I, Martinez-Menarguez JA, Castells MT, Skalaban SR, Ballesta J, Alhadeff JA, Immunocytochemical localization and biochemical characterization of a novel plasma membrane-associated, neutral pH optimal α-L-fucosidase from rat testis and epididymal spermatozoa, *Biochem J* **318**, 821–31 (1996).
9 Aminoff D, Furukawa K, Enzymes that destroy blood group specificity. I. Purification and properties of α-L-fucosidase from *Clostridium perfringens, J Biol Chem* **245**, 1659–69 (1970).
10 Bahl OP, Glycosidases of *Aspergillus niger.* I. Purification and general properties of 1,2-α-L-fucosidase, *J Biol Chem* **245**, 299–304 (1970).
11 Fukuda M, *Molecular Glycobiology* (Oxford University Press, Oxford, 1994), pp. 1–52.
12 Watkins WM, Biochemistry and Genetics of the ABO, Lewis, and P blood group systems, *Adv Hum Genet* **10**, 1–136 (1980).
13 Springer TA, Lasky LA, Cell adhesion. Sticky sugars for selectins, *Nature* **349**, 196–7 (1991).
14 Wiese TJ, Dunlap JA, Yorek MA, Effect of L-fucose and D-glucose concentration on L-fucoprotein metabolism in human Hep G2 cells and changes in fucosyltransferase and α-L-fucosidase activity in liver of diabetic rats, *Biochim Biophys Acta* **1335**, 61–72 (1997).
15 Goi G, Bairati C, Segalini G, Burlina AB, Massaccesi L, Lovagnini A, Lombardo A, Alterations in the activity of several glycohydrolases in red blood cell membrane from type 2 diabetes mellitus patients, *Metabolism* **48**, 817–21 (1999).

16 Ayude D, Fernandez-Rodriguez J, Rodriguez-Berrocal FJ, Martinez VS, de Carlos A, Gil E, Paez de La Cadena M, Value of the serum $\alpha$-L-fucosidase activity in the diagnosis of colorectal cancer, *Oncology* **59**, 310–6 (2000).
17 Inui K, Akagi M, Nishigaki T, Muramatsu T, Tsukamoto H, Okada S, A case of chronic infantile type of fucosidosis: Clinical and magnetic resonance image findings, *Brain Dev* **22**, 47–9 (2000).
18 Willems PJ, Seo HC, Coucke P, Tonlorenzi R, O'Brien JS, Spectrum of mutations in fucosidosis, *Eur J Hum* Genet **7**, 60–7 (1999).
19 Fernandez-Rodriguez J, Ayude D, de la Cadena MP, Martinez-Zorzano VS, de Carlos A, Caride-Castro A, de Castro G, Rodriguez-Berrocal FJ, $\alpha$-L-Fucosidase enzyme in the prediction of colorectal cancer patients at high risk of tumor recurrence, *Cancer Detect Trev* **24**, 143–9 (2000).
20 Sanchez-Martin MM, Cabezas JA, Ortega S, Garcia J, Garcia-Criado FJ, Pina J, Gomez-Alonso A, Levels of serum cathepsin L and several glycosidases in patients operated for colorectal cancer, *Cancer Lett* **141**, 73–7 (1999).
21 Shepherd VL, Lee YC, Schlesinger PH, Stahl PD, L-Fucose-terminated glycoconjugates are recognized by pinocytosis receptors on macrophages, *Proc Natl Acad Sci USA* **78**, 1019–22 (1981).
22 Duus JØ, Nifant'ev NE, Shashkov AS, Khatuntseva EA, Bock K, Synthesis and structural studies of branched 2-linked trisaccharides related to H type 2 blood groop determinants, *Izr J Chem* **40**, 223–39 (2000).
23 Nifant'ev NE, Amochaeva VY, Shashkov AS, Kochetkov NK, Synthesis, NMR, and conformational studies of branched oligosaccharides. 9. $\alpha$-Fucosylation by 2,3,4-tri-*O*-benzoyl-$\alpha$-L-fucopyranosyl bromide under Helferich conditions, *Carbohydr Res* **242**, 77–91 (1993).
24 Schachter H, Sarney J, McGuire EJ, Roseman S, Isolation of diphosphopyridine nucleotide-dependent L-fucose dehydrogenase from pork liver, *J Biol Chem* **244**, 4785–92 (1969).
25 Laemmli UK, Cleavage of structural proteins during the assembly of the head of bacteriophage T4, *Nature* **227**, 680–5 (1970).
26 Lowry OH, Rosenbrough NJ, Farr AL, Randall RJ, Protein measurements with the Folin phenol reagent, *J Biol Chem* **193**, 265–75 (1951).
27 Alhadeff JA, Miller AL, Wenaas H, Vedvick T, O'Brien JS, Human liver $\alpha$-L-fucosidase, *J Biol Chem* **250**, 7106–13 (1975).
28 Tsay GC, Dawson G, A sensitive spectrophotometric method for detection of L-fucose, *Anal Biochem* **78**, 423–7 (1977).
29 Leatherbarrow RT, *Enzifitter* (Elsevier Biosoft, Cambridge, 1987), p. 1.
30 Dixon M, The termination of enzyme inhibitory constant, *Biochem J* **55**, 170–1 (1953).
31 Eneyskaya EV, Golubev AM, Kachurin AM, Savel'ev AN, Neustroev KN, Transglycosylation activity of $\alpha$-galactosidase from *Trichoderma reesei*. An investigation of the active site, *Carbohydr Res* **305**, 83–91 (1998).
32 Zinin AV, Shabalin KA, Kulminskaya AA, Shishlyannikov SM, Neustroev KN, 1-*O*-Acetyl-beta-D-galactopyranose: A novel substrate for the transglycosylation reaction catalyzed by the beta-galactosidase from *Penicillium* sp., *Carbohydr Res* **337**, 635–42 (2002).
33 Vetere A, Galateo C, Paoletti S, All-aqueous, regiospecific transglycosylation synthesis of 3-*O*-$\alpha$-L-fucopyranosyl-2-acetamido-2-deoxy-D-glucopyranose, a building block for the synthesis of branched oligosaccharides, *Biochem Biophys Res Commun* **234**, 358–61 (1997).
34 Shcherbakova OG, Filatov MV, Camptothecin enhances random integration of transfected DNA into the genome of mammalian cells, *Biochim Biophys Acta* **1495**, 1–3 (2000).
35 Păhlsson P, Spitalnik SL, The role of glycosylation in synthesis and secretion of $\beta$-amyloid precursor protein by Chines hamster ovary cells, *Arch Biochem Biophys* **331**, 177–86 (1996).
36 Eneyskaya EV, Kulminskaya AA, Savel'ev AN, Shabalin KA, Golubev AM, Neustroev KN, $\alpha$-Mannosidase from *Trichoderma reesei* participates in the postsecretory deglycosylation of glycoproteins, *Biochem Biophys Res Commun* **245**, 43–9 (1998).
37 Bagiyan FG, Eneyskaya EV, Kulminskaya AA, Savel'ev AN, Shabalin KA, Neustroev KN, The action of $\alpha$-mannosidase from *Oerskovia* sp. on the mannose-rich O-linked sugar chains of glycoproteins, *Eur J Biochem* **249**, 286–92 (1997).
38 Savel'ev AN, Eneyskaya EV, Isaeva-Ivanova LS, Shabalin KA, Golubev AM, Neustroev KN, The carbohydrate moiety of $\alpha$-galactosidase from *Trichoderma reesei*, Glycoconj. J., **14**, 897–905 (1997).
39 Thompson JD, Higgins DG, Gibson TJ, Clustal W: Improving the sensitivity of progressive multiple sequence alignment through sequence weighting, positions-specific gap penalties and weight matrix choice, *Nucleic Acids Research* **22**, 4673–80 (1994).
40 Bateman A, Birney E, Cerruti L, Durbin R, Etwiller L, Eddy SR, Griffiths-Jones S, Howe KL, Marshall M, Sonnhammer EL, The Pfam Protein Families Database, *Nucleic Acids Research* **30**, 276–80 (2002).
41 Spellman MW, Leonard CK, Basa LJ, Gelineo I, van Halbeek H, Carbohydrate structures of recombinnt soluble human CD4 expressed in Chinese hamster ovary cells, *Biochemistry* **30**, 2395–406 (1991).
42 Aeed PA, Guido DM, Mathews WR, Elhamer AP, Characterization of the oligosaccaride structures on recombinnt human prorenin expressed in Chinese hamster ovary cells, *Biochemistry* **31**, 6951–61 (1992).
43 Bergwerff AA, van Oostrum J, Asselberg FA, Burgi R, Hokke CH, Kamerling JP, Vliegenthart JF, Primary structure of N-linked carbohydrate chains of a human chimeric plasminogen activator K2tu-PA expressed in Chinese hamster ovary cells, *Eur J Biochem* **212**, 639–56 (1993).
44 Withers SG, Aebersold R, Approaches to labeling and identification of active site residues in glycosidases, *Protein Sci* **4**, 361–72 (1995).
45 Henrissat B, A classification of glycosyl hydrolases based on amino acid sequence similarities, *Biochem J* **280**, 309–16 (1991).
46 Ajisaka K, Fijimoto H, Miyasato M, An $\alpha$-L-fucosidase from *Penicillium multicolor* as a candidate enzyme for the synthesis of $\alpha(1\rightarrow3)$-linked fucosyl oligosaccharides by transglycosylation, *Carbohydr Res* **309**, 125–9 (1998).
47 Opheim DJ, Touster O, The purification and characterization of rat liver lysosomal $\alpha$-L-fucosidase, *J Biol Chem* **252**, 739–43 (1977).
48 Carlsen RB, Pierce JG Purification and properties of an $\alpha$-L-fucosidase from rat epididymis, *J Biol Chem* **247**, 23–32 (1971).

Received 10 April 2002; revised 22 July 2002;
accepted 27 August 2002