α-L-Fucosidases: Exoglycosidases with Unusual Transglycosylation Properties

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Received November 18, 2003; Revised Manuscript Received March 31, 2004

ABSTRACT: α-L-Fucosidases (EC 3.2.1.51), the only members of the CAZy family GH-29, are widespread glycosidases involved in many biological processes including inflammation, metastasis, and the lysosomal storage disease fucosidosis. Despite their biological significance, information concerning the mechanism of α-L-fucosidases has only recently become available. In an attempt to obtain further data concerning their mechanism, we have investigated the hydrolytic and transglycosylation properties of a canine and a mollusk (*Pecten maximus*) α-L-fucosidase. Our results show that, despite the evolutionary distance between these two species, both enzymes have similar hydrolysis and transglycosylation properties. Surprisingly, we found that, starting from monosaccharides, these exoglycosidases were able to catalyze efficiently the synthesis of highly branched fuco-oligosaccharides as large as tetrasaccharides, a unique feature for a wild-type exoglycosidase. The structural analysis of the compounds formed revealed that the regioselectivity of α-L-fucosidases is strongly influenced by the structure of the acceptor. This leads us to propose an enzymatic approach for the preparative synthesis of fuco-oligosaccharides. This will not only allow the synthesis of biological determinants containing fucose but also of new fucose-containing oligosaccharides as α-glycosynthases appear to be difficult to obtain.

α-L-Fucosidases are exoglycosidases found in many species, from bacteria (*1*, *2*), to moulds (*3*−*7*), mollusks (*8*−*14*), and mammals (*15*−*19*). This widespread occurrence of α-L-fucosidases underlines the ubiquity and biological significance of L-fucose in living organisms. Indeed, L-fucose is one of the most common monosaccharides at the nonreducing end of many glycans (such as the Lewis series) and is thus an important biological determinant (*20*, *21*). Its terminal location makes it sensitive to the action of α-L-fucosidases, which are thus involved in many important biochemical processes such as plant defense (*22*, *23*), inflammation, metastasis, and the genetic disease fucosidosis (*21*, *24*, *25*).

Despite the biological significance of fucose metabolism, information concerning the reaction mechanism of the α-L-fucosidases has only recently become available (*26*, *27*). On the basis of their amino acid sequence similarities, all the α-L-fucosidases have been classified in a particular family (CAZy GH-29), suggesting they have a unique structure, and maybe some specific mechanistic features.

To obtain further information about the mechanism of the α-L-fucosidases, we have studied two of these enzymes, one purified from the mollusk *Pecten maximus* (*28*) and the other from a recombinant canine lysosomal α-L-fucosidase (*29*). We have analyzed their hydrolytic specificity toward various oligosaccharides and also tested their ability to synthesize various oligosaccharides by transglycosylation.

Our results demonstrate that α-L-fucosidases have the unique property, for exoglycosidases, of being able to perform the synthesis of complex oligosaccharides. Furthermore, the facile enzymatic synthesis of L-fucose-based oligosaccharides described here represents a valuable source of well-defined oligosaccharides with branched structures. These oligosaccharides are expected to lead to new insights into the biological properties of complex polysaccharides such as fucoidan (*30*).

EXPERIMENTAL PROCEDURES

Materials. The marine mollusk *P. maximus* was obtained from a local market, and its digestive glands were extracted. 2′FL,[1] 3′FL, LNFPI, and LNFPII were from IsoSep (Sweden). *p*-Nitrophenyl α-L-fucopyranoside and the various monosaccharides and methyl glycosides used were purchased from Sigma except for the methyl α-L-fucopyranoside, which was from Glycon (Germany). Other chemicals and reagents were obtained from commercial sources and were of analytical grade.

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[1] Abbreviations: 2′FL, 2′-fucosyllactose; 3′FL, 3′-fucosyllactose; LNFPI, lacto-*N*-fucopentaose I; LNFPII, lacto-*N*-fucopentaose II; *a* → *e*, axial → equatorial.

10.1021/bi036066z CCC: $27.50

6-Aminodeoxymannojirimycin was prepared as a trifluoroacetic acid salt by treatment of 6-[(*tert*-butoxycarbonyl)-amino]-1,5-imino-3,4-*O*-isopropylidene-1,5,6-trideoxy-D-mannitol (727 mg) synthesized as previously reported (*31*) with trifluoroacetic acid.

Enzyme Assays. The activity of *P. maximus* α-L-fucosidase was assayed at 60 °C for 10 min using 5 mM *p*-nitrophenyl α-L-fucopyranoside in 0.1 M sodium acetate buffer, pH 4, containing 0.2 M NaCl. The canine α-L-fucosidase was assayed under the same conditions but in 0.1 M sodium phosphate buffer, pH 7, containing 0.1 M NaCl.

The *p*-nitrophenol released was measured spectrophotometrically at 405 nm ($\epsilon = 17\,919$ at pH 9.5) using 100 μL aliquots of the reaction solution diluted with 900 μL of 0.1 M Borax buffer, pH 9.5 (*32*). One unit of α-L-fucosidase activity, as measured with the *p*-nitrophenyl α-L-fucopyranoside assay, is defined as the amount of enzyme required to release 1 μmole of *p*-nitrophenol per minute.

Large Scale Preparation of P. Maximus α-L-Fucosidase. α-L-Fucosidase from the digestive glands of *P. maximus* was purified as previously described (*28*) by three chromatographic steps, including strong cation exchange chromatography (SP-Sepharose Fast Flow (Pharmacia)), immobilized zinc affinity chromatography (Chelating Sepharose Fast Flow (Pharmacia)), and affinity chromatography on 6-aminodeoxymannojirimycin linked to Sepharose 4. Fifty milliliters of this matrix was prepared by coupling 300 mg of 6-aminodeoxymannojirimycin, as trifluoroacetic acid salt, to 50 mL of NHS-activated Sepharose 4 Fast Flow (Pharmacia), as previously described (*28*).

Usually 50 units of *P. maximus* α-L-fucosidase were applied on this affinity medium, and after addition of 50 mM of L-fucose in the elution buffer an average of 10 units of pure *P. maximus* α-L-fucosidase was obtained.

Purification of Canine α-L-Fucosidase. Recombinant canine α-L-fucosidase was purified from conditioned medium by affinity chromatography (fucosylamine agarose (Sigma)) as previously described (*29*). α-L-Fucosidase eluted with L-fucose was dialyzed against phosphate-buffered saline, concentrated to 11 mg mL^{-1} and had a specific activity of 23.5 μmol min^{-1} mg^{-1} when assayed using 4-methylumbelliferyl fucopyranoside (Sigma).

NMR Spectroscopy. NMR spectroscopy was performed on Bruker DRX-400 and DRX-600 spectrometers, operating at the proton Larmor frequency of 400.13 and 600.13 MHz, respectively, for proton observation. Spectra were recorded at 40 °C without suppression of the HOD signal. Chemical shifts are reported in ppm using acetone (δ_H 2.225 ppm, δ_C 31.07 ppm) as an internal reference.

Hydrolysis Reactions with α-L-Fucosidases Followed by NMR Spectroscopy. The exchangeable protons of the different substrates were exchanged twice with D_2O before being used for NMR experiments.

Hydrolysis in the presence of *P. maximus* α-L-fucosidase was performed in deuterated 0.1 M acetate buffer (CD_3COOD (Sigma)), 0.2 M of NaCl, pD 4.2, at 40 °C. The enzyme preparation was exchanged twice with this buffer before the experiment. For the measurement of the L-fucose anomer, the temperature was decreased to 25 °C to slow mutarotation.

For the canine α-L-fucosidase deuterated sodium phosphate buffer was prepared as follows: Na_2HPO_4 and NaCl were dissolved in D_2O, freeze-dried, and redissolved in D_2O to obtain a final concentration of 0.1 M. The pD was then adjusted to 7.2 with DCl. No prior exchange of the enzyme was necessary as the enzyme solution was highly concentrated.

Transglycosylation Reactions with α-L-Fucosidases. Transglycosylation reactions were conducted in supersaturated solutions (*33*). Briefly, to overcome the poor solubility of the donor, enough *p*-nitrophenyl α-L-fucopyranoside to give a final concentration of 70 mM was placed in a screw-capped vial along with the appropriate buffer. The vial was then heated until the components dissolved (~100 °C) and after cooling was kept at 40 °C.

For analytical experiments, the reaction was started by addition of either 2.2 U of canine, or 1 U of mollusk α-L-fucosidase, in 500 μL of a solution containing 70 mM donor and 210 mM acceptor (monosaccharide or methyl glycoside) in the appropriate buffer at 40 °C for 2 days. The reaction was monitored by ^{1}H NMR spectroscopy following the appearance of new signals in the anomeric region (δ 5.5−4.9 ppm). The presence of new compounds was also assessed by thin-layer chromatography (TLC) on silica gel plates using the solvent: ethyl acetate/methanol/acetic acid/water (12/3/3/2, V/V/V/V).

Synthesis on a preparative scale was conducted only with the canine α-L-fucosidase due to the limited availability of the mollusk enzyme. The same concentration of donor and acceptor was used but with 5 times less enzyme. α-L-Fucosidase (22 U) was dissolved with 500 mg of donor and 1 g of acceptor in 25 mL of 0.1 M phosphate buffer, pH 7. After incubation, the reaction mixture was centrifuged to remove insoluble material (20000*g*, 30 min). The supernatant was then extracted with ethyl acetate to remove the majority of the aromatic compounds. The aqueous phase was freeze-dried, redissolved in water, and fractionated using size exclusion chromatography (1 m × 2.5 cm, Bio-Gel P-2 (Bio-Rad)) with Milli-Q water as the eluent. Under these conditions, the separation of the compounds present in the reaction medium is based partly on size exclusion and partly on hydrophobic interactions. This allows the one-step removal of the aromatic derivatives and the partial separation of the oligosaccharides according to their structure.

The amount of product was determined by weighing the oligosaccharides recovered. The ratio of the various di-, tri-, and tetrasaccharides was determined by the integration of the anomeric signal arising from the fucose unit (δ_H 5.3−4.9 ppm).

RESULTS

Analysis of Substrate Specificity. P. maximus and canine α-L-fucosidases were able to hydrolyze *p*-nitrophenyl α-L-fucopyranoside according to Michaelis−Menten kinetics with K_m values of 650 μM (*28*) and 756 μM, respectively. Under our experimental conditions, the canine enzyme exhibited a maximum activity at 60 °C and had a specific activity of 44 μmol min^{-1} mg^{-1} toward *p*-nitrophenyl α-L-fucopyranoside at this temperature (data not shown).

Both enzymes are also active toward 4-methylumbelliferyl α-L-fucopyranoside (*29* and unpublished results) and are therefore considered as being "unspecific" and accordingly classified as EC 3.2.1.51.

Table 1: Hydrolysis of Different Fucosylated Oligosaccharides by the α-L-Fucosidases[a]

Oligosaccharide	Structure	% of hydrolysis[a] *P. maximus*	% of hydrolysis[a] canine
α-L-Fuc-*O*-Me	**Fucα1→**Me	0	0
2'FL	**Fucα1→2**Galβ1→4Glc	84	72
3FL	**Fucα1→3**Glc 4 ↑ Galα1	11	13
LNFP I	**Fucα1→2**Galβ1→3GlcNacβ1→ 3Galβ1→4Glc	~0	~0
LNFP II	**Fucα1→4**GlcNacβ1→3Galβ1→4Glc 3 ↑ Gal1β1	20	24

[a] 2 mM of each oligosaccharide was incubated with 0.02 units of each (canine or *P. maximus*) α-L-fucosidase in deuterated buffers at 40 °C (see Experimental Procedures). The amount of free fucose was determined by integration of the signals originating from fucose H-6 (δ 1.28−δ 1.14 ppm).

We used ^{1}H NMR spectroscopy to assess the ability of both enzymes to hydrolyze methyl α-L-fucopyranoside and several human milk fuco-oligosaccharides (Table 1). Both enzymes failed to hydrolyze methyl α-L-fucopyranoside. Furthermore, methyl α-L-fucopyranoside was found to be a very weak inhibitor as, in standard assay conditions, at 100 mM it inhibited the hydrolysis of the substrate (*p*-nitrophenyl α-L-fucopyranoside) by 32%, and 22%, for *P. maximus* and canine α-L-fucosidase, respectively.

L-Fucose was found to be a better inhibitor having a K_I of 240 and 430 μM for the *P. maximus* (*28*) and the canine enzyme, respectively. In solution, L-fucose is predominantly in the β-form (mutarotation equilibrium α/β 30/70), so either the β-form or the absence of *O*-methyl group is responsible of the inhibitory properties of L-fucose.

Among the milk fuco-oligosaccharides assayed (Table 1), both enzymes were found to be highly active toward the trisaccharide Fucα1 → 2Galβ1 → 4Glc (2′FL), but they only poorly hydrolyzed the trisaccharide Fucα1 → 3[Galβ1 → 4]Glc (3FL), indicating either increased sensitivity of the 1 → 2 linkage to hydrolysis or some steric hindrance. Nevertheless LNFPI, a pentasaccharide containing fucose at its nonreducing end, linked in α1 → 2 to galactose (as in 2′FL), was insensitive to hydrolysis. These results indicate that not only the linkage, but also the aglycon moiety, plays a key role in the interaction between α-L-fucosidases and their substrates as we had previously pointed using STD-NMR (*34*).

Determination of the Type of Hydrolysis Mechanism. Two different mechanisms have been reported for the hydrolysis reaction catalyzed by glycosidases, leading to either retention or inversion of the anomeric configuration (*35*, *36*). We thus followed the hydrolysis reaction by ^{1}H NMR spectroscopy to determine the anomeric configuration of the fucose released by the canine α-L-fucosidase. After 5 min of incubation only the α-anomer was detected (data not shown). This is direct evidence that the canine α-L-fucosidase has, like the *P. maximus* enzyme (*28*), a retaining type mechanism.

Table 2: Formation of Transglycosylated Products in the Presence of Various Acceptors and *p*-Nitrophenyl α-L-Fucopyranoside[a]

	formation of new compounds with the α-L-fucosidase from	
acceptor	*P. maximus*	canine
α-L-Fuc-*O*-Me	++++	++++
β-D-Gal-*O*-Me	++++	++++
β-D-Glc-*O*-Me	+	+
β-D-Xyl-*O*-Me	++	++
α-D-Man-*O*-Me	+	+
D-Ara	0	0
L-Fuc	0	0
none	0	0

[a] 210 mM of each acceptor was incubated with 70 mM of *p*-nitrophenyl α-L-fucopyranoside and 4 units mL^{-1} of canine α-L-fucosidase, or 2 units mL^{-1} of mollusk α-L-fucosidase in deuterated buffers at 40 °C at the appropriate pD. The symbol "++++" was used when the ratio between the intensity of the H-1 signals originating from transglycosylation products (δ 5.3−4.95 ppm) and the signal of free fucose H-1 α (δ 5.2 ppm) was ≥30%, "++" indicates a level of ≈15%, and "+" indicates a level ≤10%. The presence of transglycosylated products was also monitored by TLC.

Therefore, during the catalytic cycle, α-L-fucosidases are likely to exist as a glycosyl-enzyme intermediate, this intermediate being either intercepted by a water molecule leading to a hydrolysis reaction, or by a suitable acceptor leading to a transglycosylation reaction.

Transglycosylation Properties. To assay the transglycosylation properties of both α-L-fucosidases, and in an attempt to produce fucosylated oligosaccharides, several monosaccharides and methyl glycosides were assessed as possible acceptors (Table 2). The formation of transglycosylation products was monitored by TLC and NMR spectroscopy. Among the monosaccharides assayed, α-L-Fuc-*O*-Me and β-D-Gal-*O*-Me were found to be the best acceptors for both fucosidases. β-D-Glc-*O*-Me was a poor acceptor leading to the synthesis of four times less compounds than with β-D-Gal-*O*-Me as the acceptor. The use of the α-anomer or free glucose did not improve the yield of the transglycosylation reaction (data not shown). Xylose, which shares the same structure as glucose without C-6, was found to be a better acceptor than glucose (Table 2). While α-L-Fuc-*O*-Me is efficiently substituted, L-fucose did not allow the formation of any compounds when used as an acceptor, probably because of its inhibitory properties.

Many glycosidases are able to produce transglycosylation products only in the presence of their substrate leading to the formation of auto-condensation products (i.e., disaccharides linked to a nitrophenyl ring). In our case, we saw no evidence of such compounds, indicating that the presence of a suitable acceptor in solution is required for a transglycosylation reaction to occur.

As Fuc → Glc and Fuc → Gal are important biological determinants, and as both enzymes efficiently catalyze the synthesis of compounds with α-L-Fuc-*O*-Me as acceptor, we performed synthesis with α-L-Fuc-*O*-Me, β-D-Gal-*O*-Me, and β-D-Glc-*O*-Me on a preparative scale. On the basis of the amount of donor, we were able to obtain, after chromatographic purification on Bio-Gel P-2, transglycosylation products in yields of 13, 17, and 3%, respectively (Figure 1).

Addition of various organic cosolvents (acetone, DMSO, or poly(ethylene glycol)) and increasing the ratio of acceptor

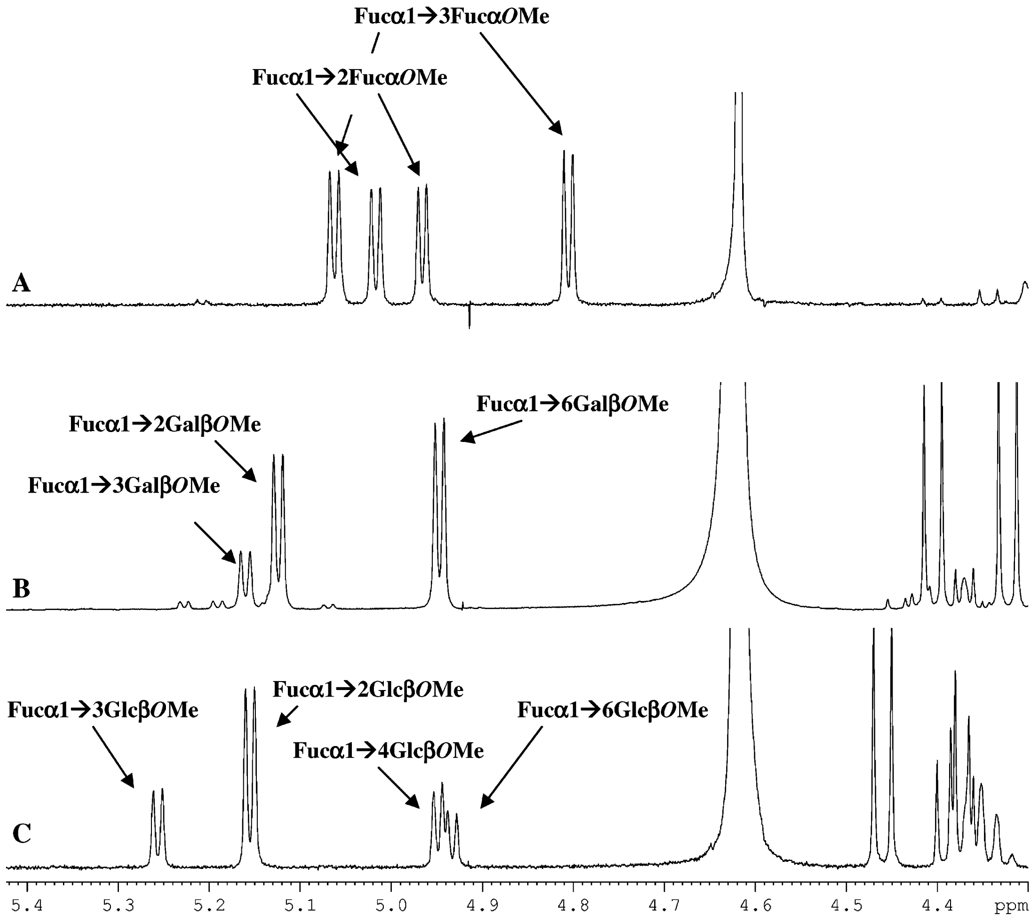

FIGURE 1: Partial ^{1}H NMR spectra (400 MHz) covering the spectral region of the anomeric protons of the disaccharides obtained by transglycosylation with (A) α-L-Fuc-*O*-Me, (B) *β*-D-Gal-*O*-Me, and (C) *β*-D-Glc-*O*-Me (D_2O at 4 °C).

to donor had no significant effect on the efficiency of the reaction.

It has been argued that the hydrolytic activity of glycosidases is enhanced in the presence of deuterium oxide (*38*). In our case, no such effect was observed as we obtained a similar ratio of hydrolysis/transglycosylation product using deuterated or aqueous buffers (measured by integration of the ^{1}H NMR spectra, data not shown).

Structural Analysis of the Oligosaccharides Obtained by Transglycosylation. When using α-L-Fuc-*O*-Me as the acceptor, only disaccharides were isolated. The structural analysis of the disaccharides, performed by standard NMR procedures (COSY, TOCSY, HSQC, HMBC), showed that both fucosidases produced L-Fuc-α1 → 2-L-Fuc-*O*-Me and L-Fuc-α1 → 3-L-Fuc-*O*-Me in a 45/55 ratio (Figure 1 and Supporting Information).

Despite the low amount of products formed with *β*-D-Glc-*O*-Me (3% yield), we decided to investigate the structure of these compounds because of their biological significance and because most published data about the transglycosylation properties of fucosidases have been obtained using either glucose or glucose derivatives as acceptors (*39*−*47*). Structural analysis of the compounds synthesized by transglycosylation of *β*-D-Glc-*O*-Me showed that a mixture of all the possible disaccharides was obtained (Figure 1 and Supporting Information). Indeed, not only α1 → 2 and α1 → 3 linked disaccharides, but also disaccharides linked in α1 → 4 and α1 → 6, were synthesized. These products were obtained with the following ratios: 45% Fucα1 → 2Glc*βO*Me, 20% Fucα1 → 3Glc*βO*Me, 20% Fucα1 → 4Glc*βO*Me, and 15% Fucα1 → 6Glc*βO*Me. Evidence of higher oligosaccharides was seen but due to the small amount of material obtained no further structural analysis was performed.

With *β*-D-Gal-*O*-Me as the acceptor, structural analysis of the compounds obtained by transglycosylation showed that a mixture of disaccharides linked α1 → 2, α1 → 3 and α1 → 6, in the ratio of 45, 42, and 13%, respectively, was obtained (see Supporting Information). As with α-L-Fuc-*O*-Me, no fucoside with a 1,4-linkage was formed. Only *β*-D-Glc-*O*-Me allows the substitution on C4 despite its poor properties as acceptor. These results show that both α-L-fucosidases only catalyze substitution on primary hydroxyl groups and on secondary hydroxyl groups in an equatorial orientation. The orientation of the C-4 hydroxyl group in *β*-D-Gal-*O*-Me and *β*-D-Glc-*O*-Me seems also to influence the ability of the fucosidases to transfer fucose to the C-6 hydroxyl group as Fucα1 → 6Glc*βO*Me is the least abundant disaccharide obtained, whereas Fucα1 → 6Gal*βO*Me is the most abundant disaccharide obtained.

In the case of D-Gal-*β*-*O*-Me, as with D-Glc-*β*-*O*-Me, evidence of higher oligosaccharides was found, but, as the transglycosylation reaction was more efficient, it was possible to perform a complete structural analysis of the products formed. ESI-MS analysis showed that we obtained, based on the molar ratios, 73% disaccharides, 20% trisaccharides, and 7% tetrasaccharides, constituting eight different products in total (Figure 2 and Supporting Information). This means that, despite the low concentration of the disaccharides

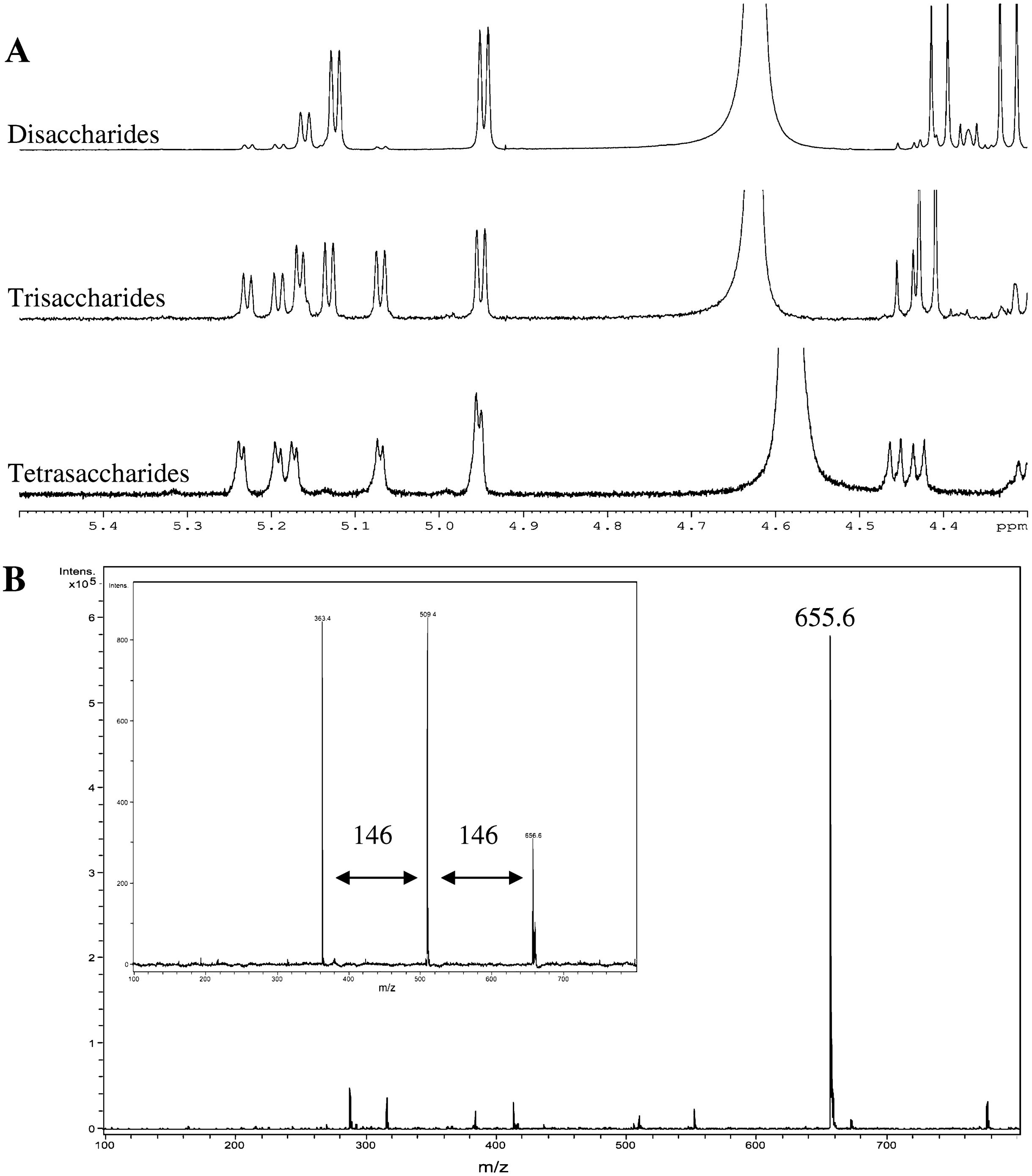

FIGURE 2: (A) Partial ^{1}H NMR spectra (400 MHz) covering the spectral region of the anomeric protons of the different fuco-galactosides obtained by transglycosylation with β-D-Gal-*O*-Me (D_2O at 40 °C), and (B) the ESI-MS analysis of the tetrasaccharide fraction $[M + Na]^+$ 655.6. The fragmentation pattern corresponding to the loss of two fucose units is represented.

formed (~8.5 mM at the end of the reaction), compared to the remaining acceptor (~200 mM), the disaccharides undergo further transglycosylation events, leading to the formation of trisaccharides (2.2 mM) and tetrasaccharides (0.84 mM).

With a very simple purification procedure, it was possible to obtain pure fractions of tri- and tetrasaccharides as indicated by ^{1}H NMR spectra (Figure 2). Furthermore, the tetrasaccharide **T-1** was initially eluted from the chromatographic step as a pure compound (Figure 3A), whereas **T-2** was obtained as a mixture with **T-1** (Figure 3B). The structural analysis of these oligosaccharides revealed that we obtained not only linear, but also branched, structures. The best example of this is **T-2**, which consisted of three fucose units linked to one galactoside unit (Figure 3B).

Each of the tri- and tetrasaccharides obtained bear fucose units with different linkage positions (Figure 4), indicating that the regioselectivity of the α-L-fucosidases is affected by the presence of a previous fucose unit. Furthermore, as in the case of the disaccharides, the tri- and tetrasaccharides were formed upon the addition of a fucose unit either on primary hydroxyl groups (C6) or on equatorial hydroxyl groups. It is worth noting that these exoglycosidases are able to produce such highly branched structures despite the probable steric hindrance that such compounds would generate. Furthermore, the structural analysis reveals that the

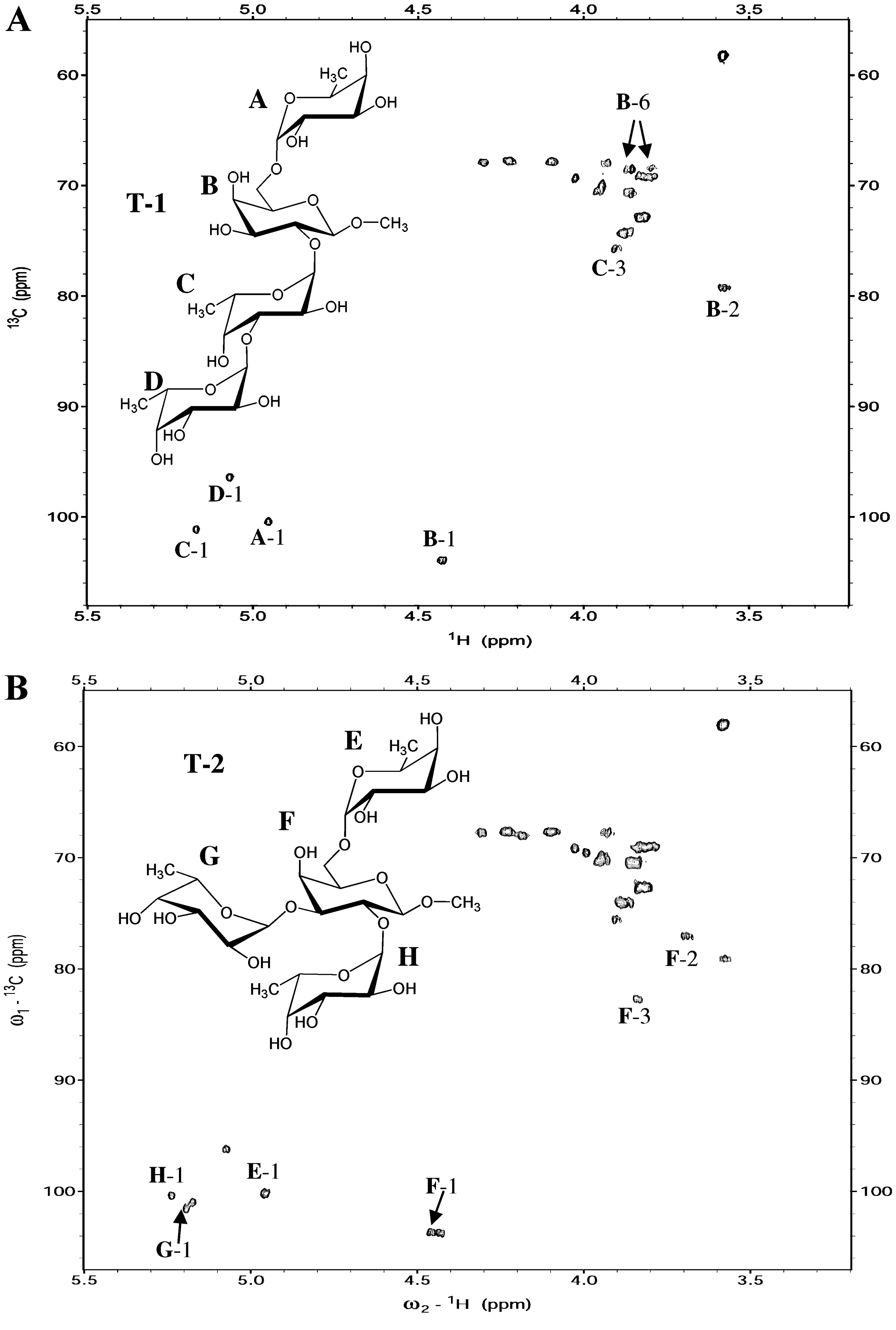

FIGURE 3: HSQC spectra of the tetrasaccharide T1 (A), and of the tetrasaccharide mixture (T1 and T2) (B) with the assignment of the relevant signals. The corresponding structures are shown in the inset (for full assignment see Supplementary Information).

disaccharide **D-1**, L-Fuc-α1 → 2-D-Gal-*β*-*O*-Me (Figure 4), plays a key role, being the only structure able to lead to all the tri- and tetrasaccharides identified.

What is apparent from our results is that the number of different structures decreases as larger structures are formed (from three di- and trisaccharides to only two tetrasaccharides) even though the number of theoretical structures increases dramatically (*48*). This indicates that the regioselectivity of the enzyme is directed by the structure of the acceptor and increases after each addition of a fucose unit. Indeed, theoretically, the trisaccharides recovered could be produced by addition of a fucose unit in position 2, 3, or 6 on the various disaccharides. However, as no evidence for the synthesis of the trisaccharide L-Fuc-α1 → 3[Fuc-α1 → 6]-D-Gal-*β*-*O*-Me was found and as all the trisaccharides bear a 1 → 2 linkage, it seems likely that all the trisaccharides

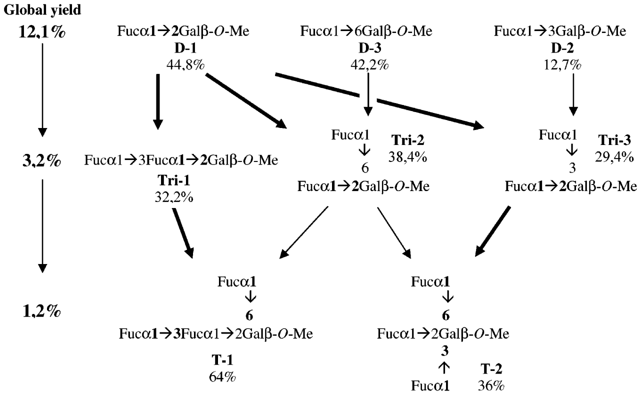

FIGURE 4: Enzymatic pathway of the fuco-galactosides. The arrows indicate the different possible pathways, and bold arrows indicate the most likely pathway. The relative yield of each compound is shown.

were derived from **D-1** by addition of a fucose unit, either in the 3- or 6-position. This hypothesis is also supported by the fact that we obtained a trisaccharide with the [Fucα1 → 3Fuc] motif (**Tri-1**) but not the [Fucα1 → 2Fuc] motif. The tetrasaccharides are then formed by the addition of a fucose unit on the 6-position of **Tri-1** and **Tri-3** and/or on the 3-position of **Tri-2** leading to compounds bearing strictly identical branches of L-fucose (i.e., α1 → 2, α1 → 3, and α1 → 6).

Hydrolytic Properties of the Fucosidases toward the Different Disaccharides. Our investigaton of the substrate specificity of both fucosidases toward fucosylated oligosaccharides from human milk (2′FL, 3FL, LNFPI, LNFPII) (Table 1) indicates that, in addition to the nature of the glycosidic bond, the aglycon moiety of the oligosaccharide is important for the interaction with the enzyme. However, because of the structural differences between these oligosaccharides, we were not able to draw clear conclusions about the linkage specificity of the fucosidases. Therefore, we used the different mixtures of isomers synthesized by transglycosylation to obtain definitive information about the hydrolytic specificity of the α-L-fucosidases.

For the fuco-galactoside and the fuco-glucoside disaccharides, the most readily hydrolyzed compounds are those that are α1 → 2 linked. In both cases, the α1 → 2 linkage is hydrolyzed 3 times faster than the other linkages (Table 3). Even though the α1 → 2 linked disaccharides are the most abundant disaccharides in both mixtures (Figures 5 and 6), under the incubation conditions used they are totally hydrolyzed within 24 h. The other disaccharides are also sensitive to hydrolysis in the following order (most to least sensitive) 1,2- > 1,3- ≥ 1,4- > 1,6- for both the fuco-galactoside and fuco-glucoside disaccharides (Table 3). The resistance to hydrolysis of the 1,6-linkage is well illustrated by the hydrolysis reaction performed on the mixture of fuco-

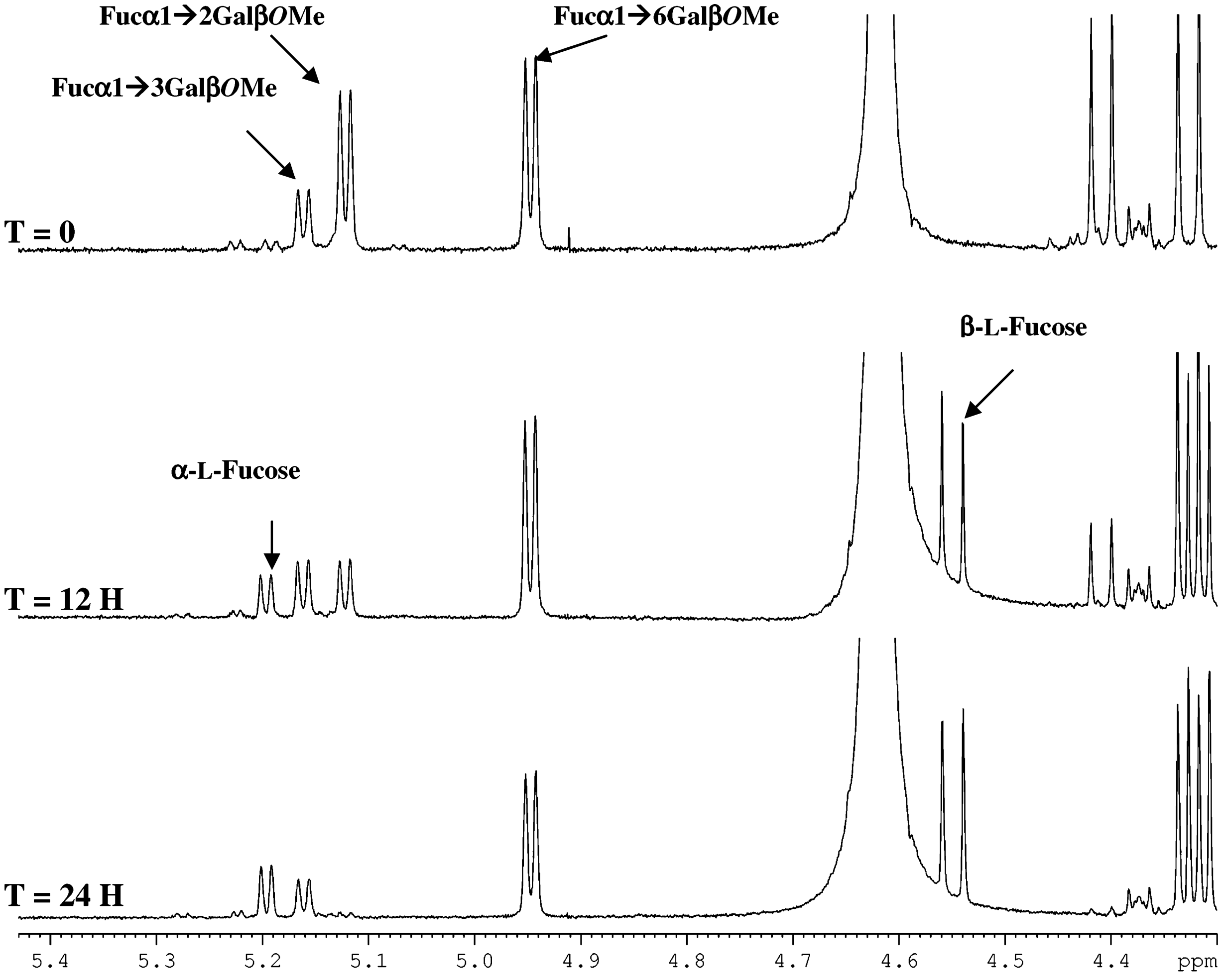

FIGURE 5: Partial ^{1}H NMR spectra (400 MHz) covering the spectral region of the anomeric protons of the fucose-galactose disaccharides incubated in the presence of canine α-L-fucosidase (deuterated phosphate buffer, pD 7.2 at 40 °C).

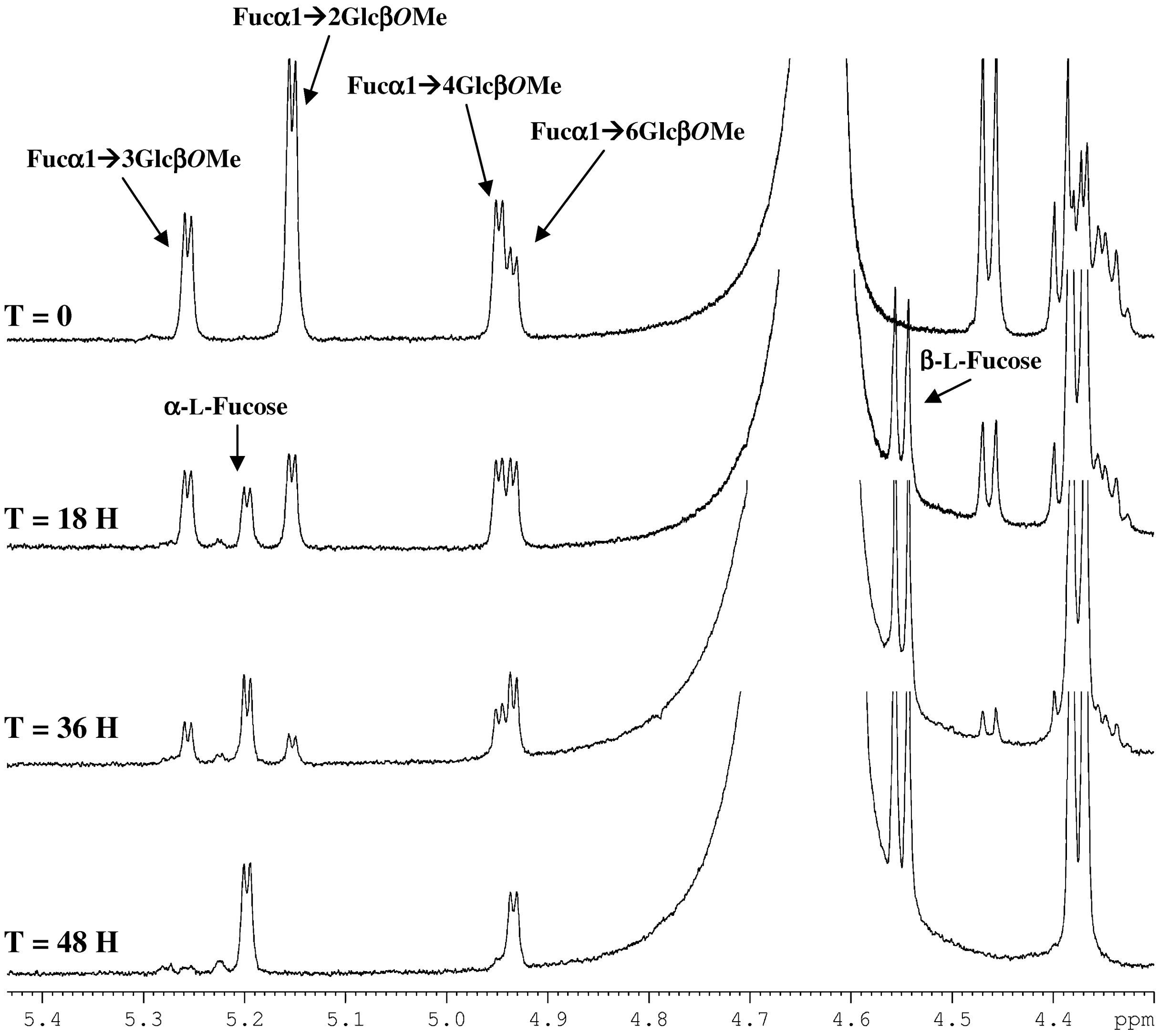

FIGURE 6: Partial ^{1}H NMR analysis (600 MHz) covering the spectral region of the anomeric protons of the fucose-glucose disaccharide incubated in the presence of canine α-L-fucosidase (deuterated phosphate buffer, pD 7.2 at 40 °C).

Table 3: Hydrolysis of Different Fucosylated Oligosaccharides by the Canine α-L-Fucosidase

disaccharide	rate of hydrolysis[a]	disaccharides	rate of hydrolysis[a]
Fucα1 → 6Glc*βO*Me	1	Fucα1 → 6Gal*βO*Me	1
Fucα1 → 4Glc*βO*Me	1.3		
Fucα1 → 3Glc*βO*Me	1.6	Fucα1 → 3Gal*βO*Me	1.3
Fucα1 → 2Glc*βO*Me	3.4	Fucα1 → 2Gal*βO*Me	3.3

[a] The rate of hydrolysis was determined by integration of the NMR signals from fucose H-1 (δ 5.25−δ 4.95 ppm) and using the signal of the 1,6-linked disaccharide as a reference. Each mixture of disaccharides was incubated with 0.02 unit of canine α-L-fucosidase in 0.1 M deuterated phosphate buffer at 40 °C (see Experimental Section).

glucoside disaccharides (Figure 6). Indeed, Fucα1 → 6Glc*βO*Me is the least abundant disaccharide in the reaction mixture at the beginning of the incubation and the only one disaccharide remaining after 48 h. This result is in agreement with the very low activity that both enzymes exhibit toward L-Fuc-α-*O*-Me, which to some extent mimics an α1 → 6 linkage.

Both fucosidases were also able to hydrolyze the fuco-disaccharides (Figure 7) but did not exhibit any specificity, hydrolyzing the α1 → 2 and α1 → 3 disaccharides equally fast. Nevertheless, hydrolysis of these disaccharides was much slower than that of the galacto- and gluco-derivatives as efficient hydrolysis required 5-fold more enzyme. Under these conditions, even after 48 h of incubation, the hydrolysis was incomplete. This probably reflects a better affinity of the aglycon binding site for glucose or galactose and would explain why we did not obtain oligosaccharides higher than disaccharides in the transglycosylation reactions with methyl α-L-fucopyranoside. Therefore, fucosidases are more active toward the αFuc → Glc and αFuc → Gal linkages, which are their natural substrates, than toward the αFuc → Fuc linkage, which has never been reported in natural products (*30*).

Several attempts have been made to link the synthetic and hydrolytic properties of the α-L-fucosidases. However, there are no simple correlations between these properties as the accumulation of transglycosylation compounds is a balance between transglycosylation and hydrolysis. Accordingly, while we obtained the same amount of Fucα1 → 2Gal-*O*-Me and Fucα1 → 6Gal-*O*-Me, this does not imply that the transglycosylation reactions forming these compounds operate with equal efficiency. Indeed, when we investigated the hydrolytic properties of the α-L-fucosidases we found that

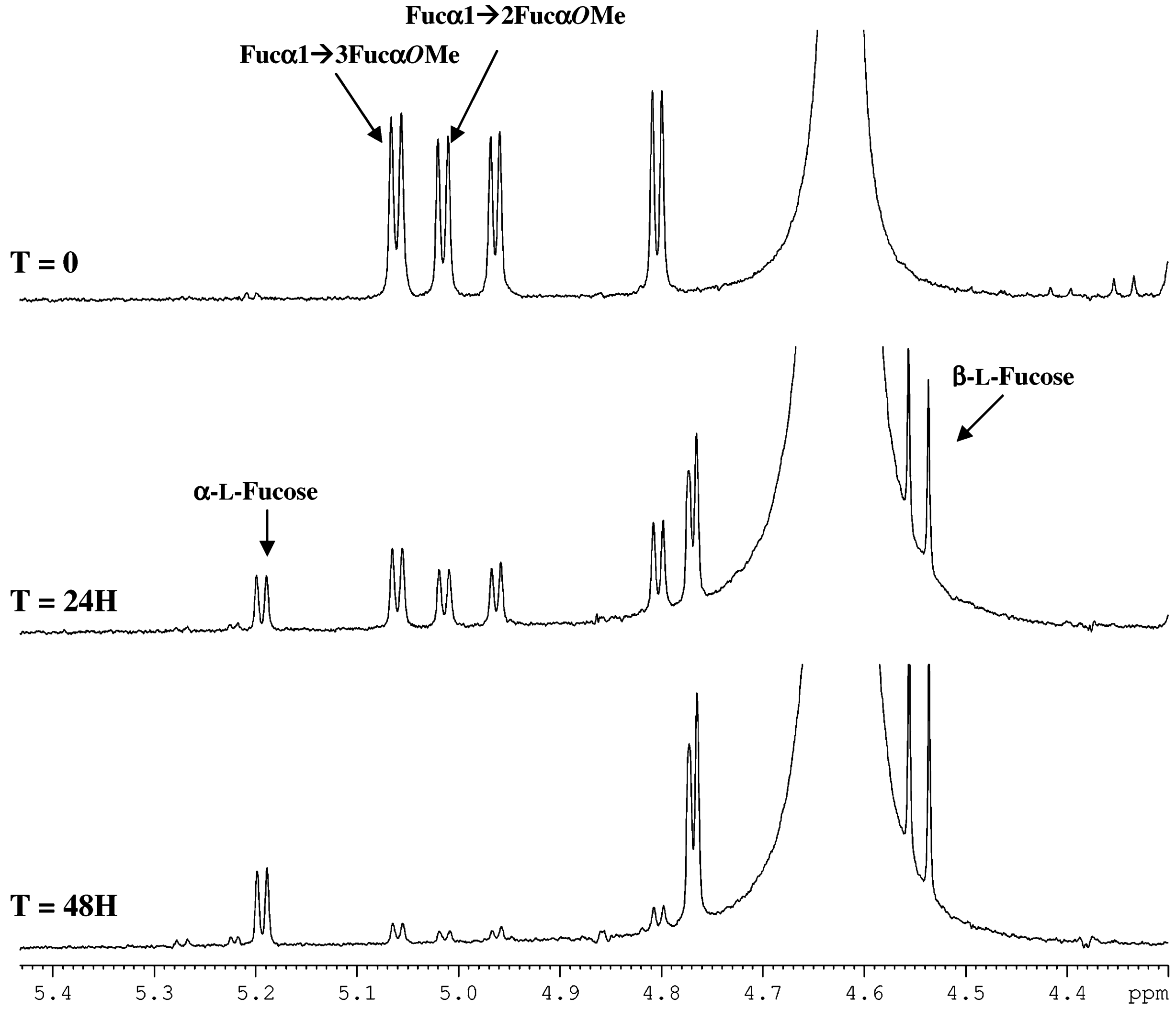

FIGURE 7: Partial ^{1}H NMR analysis (400 MHz) covering the spectral region of the anomeric protons of the fucose−fucose disaccharide incubated in the presence of canine α-L-fucosidase (deuterated phosphate buffer, pD 7.2 at 40 °C).

the 1,2-linkage is much more sensitive to hydrolysis. Therefore, to have obtained equal amounts of these two disaccharides it is likely that the efficiency of the transglycosylation reaction forming the 1,2-linkage is higher.

In an attempt to assess the influence of the size of the oligosaccharide on the hydrolysis properties of the α-L-fucosidases, we incubated the canine fucosidase with an equal amount of 2′FL and Fucα1 → 2Gal*β*-*O*-Me. Monitoring of the reaction by NMR (data not shown) demonstrated that the 2′FL was hydrolyzed slightly more rapidly than the corresponding disaccharide, despite the close proximity of a glucose unit. Therefore, it is not obvious that higher oligosaccharides are less suitable substrates and that only fucose and the penultimate monosaccharide residue are involved in the interaction with the enzyme.

DISCUSSION

The results reported in this study show that, among glycosidases, the α-L-fucosidases are a family with some particular and specific features. Our results indicate that the canine α-L-fucosidase, as all the other α-L-fucosidases investigated thus far (*26*−*28*, *37*), is a retaining enzyme, releasing only the α-anomer of L-fucose. This implies that α-L-fucosidases are likely to form a covalent glycosyl-enzyme intermediate during catalysis and thus might catalyze transglycosylation reactions.

Here we have demonstrated that both a canine and a mollusk α-L-fucosidase are able to catalyze transglycosylation reactions with the same specificity for the acceptors and for the linkages synthesized. Of particular note is that only primary hydroxyl groups and secondary equatorial hydroxyl groups are able to endow transglycosylation reactions leading exclusively to compounds with an *a* → *e* linkage configuration. Furthermore, the α1 → 2 linkage appears to be preferentially formed as disaccharides with this linkage are either the, or one of the, major disaccharides formed, and this linkage is present in all the tri- and tetrasaccharides recovered.

The ability of some α-L-fucosidases to catalyze such reactions has been demonstrated previously. To date, two mammalian (*17*, *41*, *43*, *44*), one mollusk (*39*), two moulds (*39*, *40*, *42*, *45*), two bacterial (*39*, *47*), and one archae bacterial (*46*) α-L-fucosidase, have been investigated. Despite the various conditions used (i.e., the addition of organic solvents, the donor/acceptor ratio, the amount of enzyme), the results obtained in these studies were very similar to ours. Notably, all the oligosaccharides produced contained only α-linkages, providing further evidence for a retaining type mechanism for the α-L-fucosidases.

Furthermore, all these α-L-fucosidases catalyze the formation of a 1 → 6 bond or (and) the formation of a glycosidic bond with an *a* → *e* configuration. No one has reported the transfer of fucose to C-4 of galactose, using enzymes of eukaryotic or prokaryotic origin, confirming the selectivity of α-L-fucosidases for equatorial secondary hydroxyl groups.

Nevertheless, our work is the first report of α-L-fucosidases able to produce oligosaccharides higher than disaccharides. Even if one α-L-fucosidase has been used for the synthesis of trisaccharides (*47*), it was only by the addition of one fucose unit onto a disaccharide acceptor. Our results show unambiguously that at least eukaroytic α-L-fucosidases are able to synthesize oligosaccharides up to tetrasaccharides, starting from monosaccharides. The enzymes predominantly catalyze the addition of fucose onto the galactose unit leading to highly branched oligosaccharides, which would be challenging to obtain by total synthesis. To the best of our knowledge, no (wild-type) exoglycosidases have been reported to catalyze the synthesis of branched oligosaccharides and only one thermophilic exoglycosynthase with branching properties has been reported (*49*).

These results indicate that both fucosidases might have higher affinity for the higher oligosaccharides. Indeed, despite the low concentration of di- and trisaccharides obtained, at the end of the reaction both α-L-fucosidases catalyze further substitutions on these oligosaccharides rather than on the unsubstituted acceptor, which is 20−50-fold more abundant. This leads us to propose a previously undescribed enzymatic approach for the synthesis of various biological determinants and novel branched fuco-oligosaccharides. Furthermore, it seems likely that the properties we describe here will be found with other eukaryotic fucosidases as we obtained identical results using a mammalian and a mollusk enzyme.

In conclusion, our results show that α-L-fucosidases have common properties, which are in agreement with their CAZy classification in one family. Under our experimental conditions, it was possible to perform the synthesis of various and complex oligosaccharides with new structures on a preparative scale. This represents a valuable approach as the α-glycosynthase approach is still in its infancy (*50*) and an attempt to develop a fucosynthase was not successful (*26*).

ACKNOWLEDGMENT

The European Network Glycotrain (HPRN-CT2000-00001) and a NH&MRC Australian program grant supported this work.

SUPPORTING INFORMATION AVAILABLE

Chemical shifts of the transglycosylation reactions products. This material is available free of charge via the Internet at http://pubs.acs.org.

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BI036066Z