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Characterization of a new α-L-fucosidase isolated from the marine mollusk *Pecten maximus* that catalyzes the hydrolysis of α-L-fucose from algal fucoidan (*Ascophyllum nodosum*)

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Algal fucoidan is an α-L-fucose-based polysaccharide endowed with important biological properties for which the structure has not yet been fully elucidated. In an attempt to implement new enzymatic tools for structural study of this polysaccharide, we have found a fucosidase activity in the digestive glands of the common marine mollusk *Pecten maximus*, which is active on a fucoidan extracted from the brown algae *Ascophyllum nodosum*. We now report the purification and characterization of this α-L-fucosidase (EC 3.2.1.51). The enzyme was purified by three chromatographic steps, including an essential affinity chromatography based on the glycosidase inhibitor analog 6-amino-deoxymannojirimycin as the ligand. The purified α-L-fucosidase is a tetrameric glycoprotein of 200 kDa that hydrolyzes the synthetic substrate *p*-nitrophenyl α-L-fucopyranoside with a K_m value of 650 μM. This enzyme has high catalytic activity (85 μmol · min^{-1} · mg^{-1}) compared with the other known fucosidases and also possesses an unusual thermal stability. The purified α-L-fucosidase is a retaining glycosidase. The activity of the purified fucosidase was determined on two structurally different fucoidans of the brown algae *A. nodosum* and *Fucus vesiculosus* to delineate glycosidic bond specificity. This report is to our knowledge the first demonstration of a fucosidase that can efficiently release α-L-fucose from fucoidan.

Key words: 6-*N*-acetylated-deoxymannojirimycin/affinity chromatography/α-L-fucosidase/fucoidan

Introduction

Algal fucoidan from brown algae (e.g., *Ascophyllum nodosum* and *Fucus vesiculosus*) is a sulfated polysaccharide endowed with important biological properties in several mammalian systems: fucoidan is anticoagulant and antithrombotic via the activation of both antithrombin and heparin cofactor II (Nagumo and Nishino, 1996), anti-inflammatory (Angstwurm *et al.*, 1995; Preobrazhenskaya *et al.*, 1997), antitumoral (Soeda *et al.*, 1994), contraceptive by inhibiting penetration of the human zona pellucida (Huang *et al.*, 1982), and antiviral, as reported in the case of human cell invasion by retroviruses including human immunodeficiency virus (McClure *et al.*, 1992). A major hindrance to the development of its therapeutic application is the poor knowledge of its molecular structure and, as a consequence, of the structural basis of its biological properties (Mulloy *et al.*, 2000). Polysaccharide-degrading or -modifying enzymes could seriously improve our knowledge of its structure.

In the emerging field of polysaccharide sequencing, a major interest is raised by glycosidases that, because of their enzymatic selectivity, are helpful tools to study sequence-function relationships (Turnbull *et al.*, 1999). Given the algal origin, we have looked for such enzymes in marine environments and have found that the marine mollusk *Pecten maximus* was a rich source of glycosidases (Daniel *et al.*, 1999). To our knowledge no fucosidase active on *A. nodosum* fucoidan has been previously described. We report here the first purification of an α-L-fucosidase from the digestive glands of *P. maximus* able to release fucose from fucoidan. The physicochemical and catalytic properties of the fucosidase were determined, and its action on fucosylated oligosaccharides and on *A. nodosum* and *F. vesiculosus* fucoidans was analyzed by pulsed amperometric detection of the released saccharides.

Results

Isolation of α-L-fucosidase from P. maximus

Purification of the α-L-fucosidase was achieved through three chromatographic steps including the essential affinity chromatography based on the glycosidase inhibitor 6-*N*-acetylated-deoxymannojirimycin (DMJ; compound **1**, Figure 1). Indeed, all our attempts to purify the α-L-fucosidase with only standard commercially available chromatographic media were unsuccessful, including the affinity medium agarose-ε-aminocaproylfucosamine. This matrix was unable to bind *P. maximus* α-L-fucosidase and led us to prepare our own affinity medium based on 6-*N*-acetylated-DMJ. We found that this imino-sugar was a competitive inhibitor of *P. maximus* α-L-fucosidase (see the following), and we took advantage of this property to use the amino derivative as a valuable affinity ligand (compound **2**, Figure 1).

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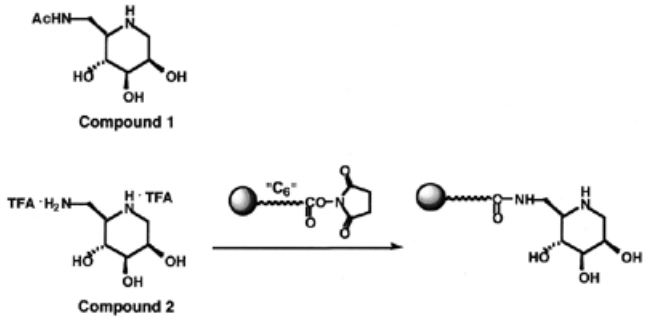

Fig. 1. Structure of 6-*N*-acetylated-DMJ (compound **1**) and 6-*N*-amino-DMJ (compound **2**). Compound **2** was coupled to NHS-activated chromatographic support to serve as an affinity ligand for purification of fucosidase.

Table I summarizes the purification of α-L-fucosidase from the digestive glands of *P. maximus*. After extraction and fractionated precipitation with ammonium sulfate, lipid material was removed from the protein extract with a subsequent wash with ethyl-acetate (30% v/v). Then the extract was applied to a SP-Sepharose cation exchanger equilibrated at pH 5.25, on which the fucosidase activity was retained and afterward eluted by 0.1 M NaCl (Figure 2A). A second step on an immobilized zinc affinity column allowed elimination of a large part of inactive proteins without substantial loss of activity and resulting in a good purification of the enzyme. The bound α-L-fucosidase was easily eluted by adding 10 mM imidazole in the elution buffer (Figure 2B). This result indicates the high capability of the enzyme to form coordination complexes with immobilized zinc ions, likely through histidine residues of the protein surface. The purification protocol was completed with an affinity chromatographic step on 6-*N*-acetylated-DMJ–Sepharose prepared as described in *Materials and methods*. The enzyme bound strongly on this medium and was specifically eluted by 50 mM fucose (Figure 2C). The enzyme was purified with a factor of 570-fold and migrated on a sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) (12.5%) as a single species.

Physicochemical properties of the purified fucosidase

The molecular weight of the enzyme was determined to be 50,000 by denaturing gel electrophoresis (Figure 3) and 200,000 by gel filtration on Superose 12. This result suggests that the native enzyme is a tetramer composed of essentially identical subunits as the wide majority of the previously described fucosidases (Cabezas *et al.*, 1983; Johnson and Alhadeff, 1991). The pI of the highly purified fucosidase determined by isoelectrofocusing was estimated to be 6.3. This was confirmed by the activity staining of the IEF gel, which showed fucosidase activity at the same pI. SDS–PAGE stained with periodic acid–Schiff reagent indicated that the purified α-L-fucosidase was glycosylated. Furthermore, treatment with *N*-glycosidase F gave a sharper band, which is not stained with periodic acid–Schiff reagent and exhibits a slightly lower mass (Figure 4). In connection to this glycosylation, it is worthwhile to note that the enzyme is bound very tightly to a concanavalin A–Sepharose medium (Pharmacia) and cannot be eluted unless using denaturing conditions. The bound enzyme remained active on this medium (data not shown), indicating a possible use as an immobilized enzyme.

Functional properties

The enzyme assayed with *p*-nitrophenyl–α-L-fucoside as substrate exhibited an optimal pH at 4.0. More than 50% of the fucosidase activity was maintained at pH 3.0 and 5.5. Maximal enzyme activity was achieved at 60°C. In these optimal conditions of temperature and pH (fucosidase 25 $\mu g \cdot ml^{-1}$, pH 4) 40% of the activity remained after 1 day at 60°C (Figure 5). At temperatures up to 30°C the activity was stable over a long period of time because 70% remained after 40 days at 30°C. It is significant that the enzyme activity increased with the ionic strength, as observed from the twofold enhancement of the reaction rate with an increase in the ionic strength from 10 mM to 200 mM. All the data show a high stability that is uncommon among previously described fucosidases.

The sensitivity of fucosidase to the metal ions Ca^{2+}, Mg^{2+}, Mn^{2+}, Co^{2+}, Zn^{2+}, Cu^{2+}, Hg^{2+}, at various concentration (from 0.1 to 5 mM) was measured with *p*-nitrophenyl–α-L-fucoside as substrate in 0.1 M acetate buffer, pH 4. These results showed that the enzyme is slightly inhibited by Cu^{2+} and strongly by Hg^{2+}, which may indicate the involvement of sulfhydryl groups in the catalytic activity (Table II). The other metal ions tested are moderate activators even at low concentrations (100 μM). The most efficient metal was Zn^{2+}, which increased the enzyme activity by 150% at 1 mM. These results suggest a possible stabilization of the tetramer with divalent cations. Ethylenediamine tetra-acetic acid (EDTA) assayed in the same concentration range also behaves as a moderate activator. These properties are comparable only to those observed for the α-L-fucosidase

Table I. Purification of α-L-fucosidase from the digestive glands of *Pecten maximus*

Purification steps	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Recovery (%)	Purification
Crude	1180	177	0.15	100	1
Extraction	787.7	165.4	0.21	93	1.4
30%-70% $(NH_4)_2SO_4$ precipitate	514.3	144	0.28	81.5	1.8
Ethyl-acetate wash 30%	91.35	74	0.81	42	5.35
Strong cation axchange chromatography	8	24	3	15	19.6
Immobilized zinc affinity chromatography	0.761	16	21	9.6	139
Affinity chromatography	0.070	6	86	3.4	568

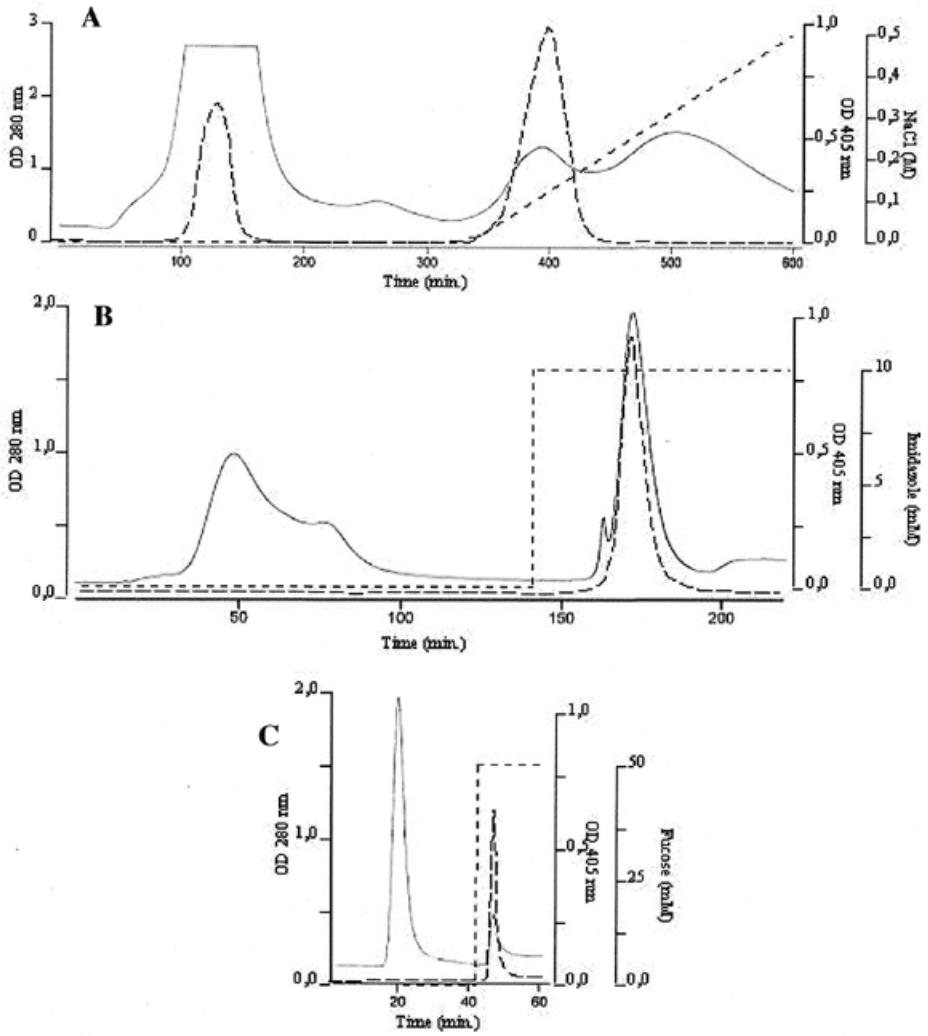

Fig. 2. Chromatographic purification of the α-L-fucosidase by strong cation exchange chromatography (**A**), followed by immobilized zinc affinity chromatography (**B**) and affinity chromatography (**C**). The α-L-fucosidase activity was measured at 405 nm on the synthetic substrate *p*-nitrophenyl–α-L-fucose as described in *Materials and methods* (large dotted lines). Smaller dotted line indicates the various gradients of NaCl, imidazole, and fucose of the chromatographic steps (**A**), (**B**), (**C**), respectively (see *Materials and methods*).

from the marine mollusk *Chamelea gallina* (Reglero and Cabezas, 1976), whereas cations are generally described as weak α-L-fucosidase inhibitors.

Enzymatic hydrolysis of glycosidic bonds is known to occur via two general mechanisms leading either to retention or to inversion of the anomeric configuration (Koshland, 1953). To determine which of these two mechanisms was operating with the α-L-fucosidase, we followed the enzymatic hydrolysis of *p*-nitrophenyl–α-L-fucose by ^{1}H nuclear magnetic resonance (NMR) and identified the anomeric configuration of the fucose produced. Prior to enzymatic reaction, mutarotation kinetics of α-L-fucose in deuterated acetate buffer was followed at 25°C. Mutarotation equilibrium (ratio α/β 30/70) was reached after 40 min in solution, showing that the β-L-fucose is the major form of L-fucose in solution.

The NMR spectrum of *p*-nitrophenyl–α-L-fucose exhibited an anomeric proton H-1-α signal at 5.82 ppm and H-6 signal at 1.16 ppm. When incubated at 25°C with enzyme extract in the NMR tube, ^{1}H NMR spectra revealed a continuous decrease in the signals originating, respectively, from H-1 and H-6 of *p*-nitrophenyl–α-L-fucose. Characteristic signals of free fucose appeared and increased concomitantly. At the beginning of reaction the α anomer was the first to be released, while β-L-fucose appeared just after and later became the major species because of the mutarotation. After 7 min of reaction, free fucose appeared with an anomeric ratio α/β of 80/20. After 30 min of reaction, this ratio α/β was 60/40. This result clearly shows that the hydrolytic mechanism of *P. maximus* α-L-fucosidase proceeds with retention of configuration.

Kinetic and inhibition studies

Using the synthetic substrate *p*-nitrophenyl–α-L-fucopyranoside, we showed that the kinetics of *P. maximus* fucosidase could be described according the Michaelis-Menten model with a K_m value of 650 μM. Several glycosidase inhibitors have been tested (Table III) including imino-sugars, fucose, and fucosamine. The K_i for fucosamine is 316 μM. This value could explain the unsuccessful trials of purification on

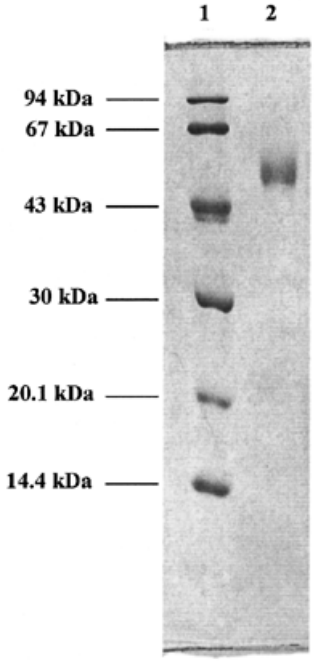

Fig. 3. SDS–PAGE (12.5%) of purified α-L-fucosidase (lane 2). Molecular weight markers are indicated on lane 1.

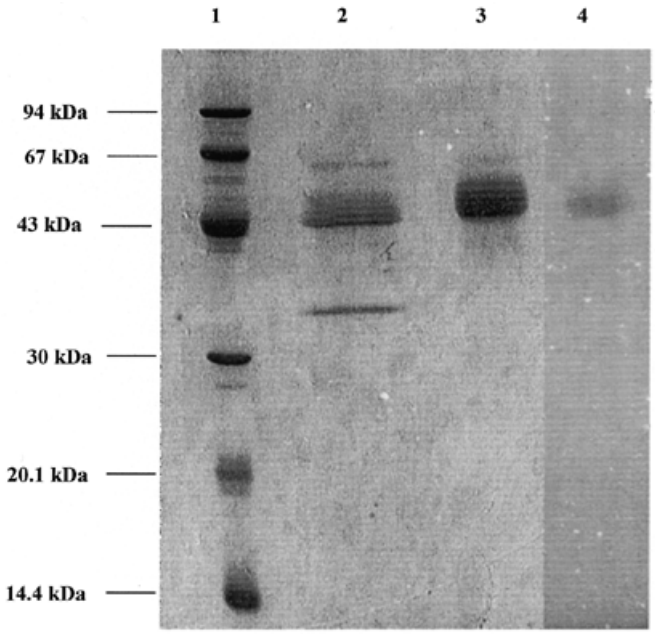

Fig. 4. SDS–PAGE (12.5%) of the purified α-L-fucosidase (lane 3) after coloration with periodic acid–Schiff reagent (lane 4) or N-glycosidase F treatment (lane 2). Molecular weight markers are indicated on lane 1.

agarose-ε-aminocaproylfucosamine matrix. The reaction product fucose is a better inhibitor than fucosamine, whereas glycosamine derivatives are frequently reported to be better inhibitors (10–100 times) than their corresponding carbohydrate (Legler, 1990). Commercial 1-DMJ is a slightly more potent inhibitor (K_i 34 μM) than 6-*N*-acetylated-DMJ with a K_i value of 55 μM. As it has been observed with the other purified fucosidases, the most efficient inhibitor is deoxyfuconojirimycin with an inhibition constant K_i of 26 nM, comparable to the K_i value for bovine and human α-L-fucosidases (2.7 and 10 nM, respectively) (Fleet *et al.*, 1985; Legler *et al.*, 1995).

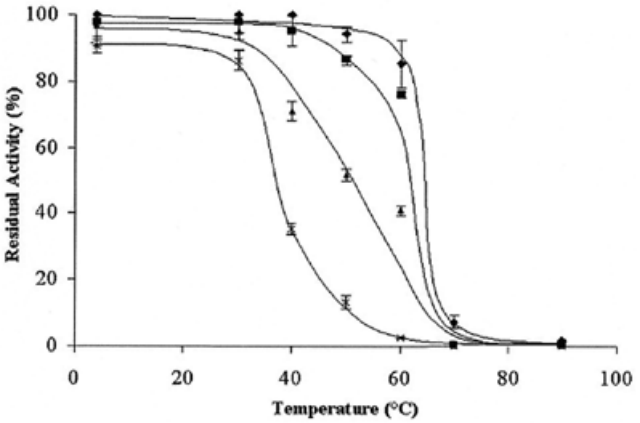

Fig. 5. Dependence on temperature of the α-L-fucosidase activity. The enzyme was incubated for several days at 0.025 mg/ml in 0.1 M acetate buffer, pH 4, at different temperatures (from 4°C to 90°C). Residual activity assayed on the synthetic substrate *p*-nitrophenyl–α-L-fucose was measured at different times:10 min (squares), 1 h (diamonds), 1 day (triangles), and 7 days (x's).

Table II. Action of effectors (1 mM) on α-L-fucosidase activity

Compounds	Relative activity[a] (%)
Hg^{2+}	10
Cu^{2+}	88
Ca^{2+}	122
Mg^{2+}	130
Mn^{2+}	132
Co^{2+}	134
Zn^{2}	146
EDTA	141

[a]The activity was measured at 60°C for 10 min in 0.1 M acetate buffer, pH 4, containing 3.5 mM *p*-nitrophenyl–α-L-fucopyranoside as substrate and was compared to the activity measured without effector (100%).

Table III. Inhibition of the α-L-fucosidase by L-fucose and nitrogen containing glycosides

Inhibitors	K_i
1-Deoxygalactonojirimycin	>1 mM
1-Deoxynojirimycin	>1 mM
L-Fucosamine	316 μM
L-Fucose	240 μM
6-*N*-Acetylamino DMJ	55 μM
1-DMJ	34 μM
1-Deoxyfuconojirimycin	0.026 μM

The assays were performed with *p*-nitrophenyl–α-L-fucopyranoside as substrate as described under *Materials and methods*.

Substrate specificity

The ability of the α-L-fucosidase to hydrolyze different *p*-nitrophenyl glycosides has been assayed spectrophotometrically. Only α-L-fucospyranoside was hydrolyzed among the 16 different glycosides tested (see *Materials and methods*) even if the incubation time was extended up to 90 min. Therefore our enzyme preparation was free from other glycosidase activities, such as galactosidase and glucosidase, abundant in the digestive glands extract of *P. maximus* (Daniel *et al.*, 1999).

In addition, when the α-L-fucosidase was incubated with several milk oligosaccharides (1 mM) containing fucose at different linkage positions, no other monosaccharide than fucose was detected, even after 1 day of incubation and in spite of the sensitivity of the amperometric detection used. The Fuc α(1→2)Gal linkage in the trisaccharide 2′-fucosyllactose (2′FL) was the most readily hydrolyzed. Among the pentasaccharides assayed, the Fucα(1→4)GlcNac linkage in lacto-N-fucopentaose (LNFP) II was the most sensitive to the hydrolysis (Table IV). The Fucα(1→3)GlcNac linkage in LNFP III and the Fucα(1→2)Gal linkage in LNFP I were quite insensitive to hydrolysis (around 5%). Regarding these results, no simple relationships can be drawn, but it is likely that the surroundings of the glycosidic bond strongly influences the α-L-fucosidase activity. The high rate of hydrolysis especially concerning 2′FL and LNFP II were obtained with very low amounts of fucosidase (0.025 U), indicating a high activity of the fucosidase toward these fucosylated oligosaccharides.

Activity on fucoidan

Fucosidase activity was tested on two fucoidans extracted from the two different brown algae *A. nodosum* and *F. vesiculosus*, which have been described to exhibit structural differences. Thus, 0.5 U purified α-L-fucosidase was incubated with 60 mg of each fucoidan in 5 ml of 50 mM acetate buffer, pH 5, at 40°C. These conditions, which slightly differ from the optimal conditions determined for the hydrolysis of the synthetic substrate, were chosen to prevent acid-catalyzed depolymerization of the fucoidan and to facilitate the amperometric detection. In another set of experiments, digestions were performed in the presence of the α-L-fucosidase (0.5 U) and a partially purified sulfatase (0.5 U) that we have recently reported to be active on the *A. nodosum* fucoidan (Daniel *et al.*, 2001). Under these conditions fucose was released only from *A. nodosum* fucoidan in presence of the α-L-fucosidase or the combination of α-L-fucosidase and sulfatase as determined by high-performance liquid chromatography (HPLC)–pulsed amperometric detection (PAD) analysis. Approximately 2 µmoles of fucose were released after 5 days of incubation with fucosidase. Hydrolysis was increased by 75% (3.5 µmoles) in presence of sulfatase, indicating that both enzymes may act synergistically to hydrolyze *A. nodosum* fucoidan (Figure 6). The low amount of released fucose (2.3% of the total fucose available) corresponds to few fucosyl units hydrolyzed per molecule of polysaccharide, and therefore it did not lead to a significant decrease of the molecular weight of polysaccharide as determined by HPLC–size exclusion chromatography (SEC) (data not shown). We assume that the α-L-fucosidase hydrolyzes unsulfated fucose linked either at the nonreducing end or at a branch point of the polysaccharide. The increase in fucose released when the sulfatase is added may result from desulfation of sulfated fucose catalyzed by the sulfatase, making available additional fucose to the α-L-fucosidase activity. It is significant that no

Table IV. Extent of fucose hydrolyzed from fucosylated oligosaccharides by the α-L-fusocidase

Oligosaccharide	Structure	% of hydrolysis[a]	
		after 6 hours of incubation	after 24 hours of incubation
2'-FL	**Fucα1→2**Galβ1→4Glc	95.6	100
LNFP I	Galβ1→3GlcNacβ1→3Galβ1→4Glc **2** ↑ **Fucα1**	5.4	6.4
LNFP II	Galβ1→3GlcNacβ1→3Galβ1→4Glc **4** ↑ **Fucα1**	28.5	57.7
LNFP III	Galβ1→4GlcNacβ1→3Galβ1→4Glc **3** ↑ **Fucα1**	6	7.8

[a]1 mM of each oligosaccharide was incubated with 0.025 U of α-L-fucocisadase in 0.1 M acetate buffer, pH 4, at 60°C. The amount of fucose was determined by HPLC-PAD as described under *Materials and methods*.

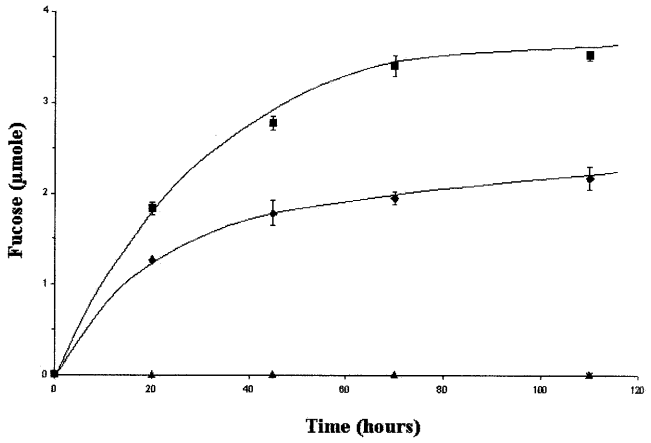

Fig. 6. Hydrolysis of fucose from fucoidan catalyzed by the α-L-fucosidase. *A. nodosum* fucoidan (60 mg) was incubated in 5 ml of 50 mM acetate buffer, pH 5, at 40°C with fucosidase alone (0.5 U) (diamonds) or with both fucosidase and sulfatase (0.5 U each) (squares). Controls corresponded to incubation of fucoidan alone (x's) or in presence of sulfatase (0.5 U) (triangles) in the same buffer and temperature conditions. The amount of fucose was determined by HPLC-PAD as described in *Materials and methods*.

fucose was detected when the α-L-fucosidase was incubated with the fucoidan from *F. vesiculosus*, even in the presence of sulfatase, even though the α-L-fucosidase remained active throughout the incubation (data not shown).

Discussion

We have previously reported that the digestive gland extract from *P. maximus* is rich in numerous glycosidases, some of them endowed with a fucoidan-degrading activity (Daniel *et al.*, 1999). Here we report the first purification and characterization of an α-L-fucosidase able to catalyze the hydrolysis of fucosyl units of *A. nodosum* fucoidan. The purification scheme involves affinity chromatography based on an imino-sugar. Imino-sugars initially found in microorganisms and plants are very potent glycosidase inhibitors (Legler, 1990). Their inhibitory capacity relies on their structural analogy with the activated form of the corresponding sugar, as they are thought to mimic of the oxocarbonium transition state produced during the catalytic hydrolysis reaction (Sinnott, 1990). DMJ is a more potent inhibitor of α-L-fucosidase than α-mannosidase (Evans *et al.*, 1985). Accordingly it has previously been reported that 6-*N*-acetylamino-DMJ (compound **1**) was a specific inhibitor of α-L-fucosidase from bovine kidney (McCort *et al.*, 2000). The common structural feature to all inhibitors of α-L-fucosidase is the correct stereochemistry of the three hydroxyl groups on the piperidine ring corresponding to C-2, C-3, and C-4 of L-fucose (Winchester *et al.*, 1990; Asano *et al.*, 2001). Compound **1** has the minimum structural requirement of DMJ. The modification at C-6 does not impair the affinity toward α-L-fucosidase, as seen in Table III. As compound **1** shows moderate inhibition toward α-L-fucosidase in *P. maximus*, the amino group at C-6 of the imino-sugar constitutes a useful anchoring group for immobilization on polymer supports for chromatographic affinity purification. Indeed, the primary amine linked to the resin restores the amide bond important for the inhibition.

The purified enzyme, free from sulfatase and other glycosidases activities, is a tetrameric glycoprotein of 200 kDa. These structural features are shared by most of purified fucosidases in mammals (mouse, rat, human, pig, bovine, monkey) (Johnson and Alhadeff, 1991), mollusks (*Charonia lampas*, *Venus merceneria*, *Chamelea gallina*, *Tapes rhomboideus*, *Littorina littorina*, *Unio elongatulus*) (Cabezas *et al.*, 1983; Focarelli *et al.*, 1997) and bacteria (*Clostridium perfringens*) (Aminoff and Furukawa, 1970). However, purified enzyme differs in many aspects. Its specific activity of 85 $\mu mol \cdot min^{-1} \cdot mg^{-1}$ is one of the highest reported, second only to the α-L-fucosidase from *Fusarium oxysporum* (110 $\mu mol \cdot min^{-1} \cdot mg^{-1}$) (Yamamoto *et al.*, 1986). A second interesting characteristic is its stability in relatively acidic medium (pH 4) and high temperatures (above 50°C). Furthermore, in contrast with the other described fucosidases, *P. maximus* fucosidase is stable at room temperature for several weeks (30% remaining activity after 1 year at room temperature), and it can be freeze-dried without any particular preservative treatment and without significant loss of activity. NMR analysis of the anomeric configuration of the hydrolysis product of the α-L-fucosidase indicates that this enzyme retains the anomeric bond configuration, producing α-anomers that progressively give rise to β-anomers when mutarotation takes place. The enzyme belongs therefore to the class of "retaining enzymes" (Sinnott, 1990; Withers, 2001). Its thermal stability and its high specific activity make the purified α-L-fucosidase a valuable tool in biocatalysis.

Among the different imino-sugars tested, the most potent is 1-deoxyfuconojirimycin (K_i = 26 nM) as for most α-L-fucosidases described (Robina *et al.*, 2001). However, the purified enzyme is about 1000 times less sensitive than many other α-L-fucosidases to 1-DMJ. As an example, the human liver α-L-fucosidase is equally as sensitive toward 1-deoxyfuconojirimycin as toward 1-DMJ (Fleet *et al.*, 1985). This result suggests a high specificity of the purified enzyme. Except for *p*-nitrophenyl–α-L-fucopyranoside, none of the synthetic glycosides tested (see *Materials and methods*) were hydrolyzed. In agreement with this specificity, only fucose was released on incubation of α-L-fucosidase with fucosylated oligosaccharides. No other monosaccharide (glucose or galactose) were detected despite the sensitivity of the analytical technique used (PAD).

Though the purified enzyme is specific for α-L-fucose, this fucosidase does not exhibit a clear linkage specificity based on the results obtained with fucosylated oligosaccharides. The linkage environment seems to strongly influence the rate of hydrolysis. Indeed, the purified α-L-fucosidase efficiently cleaved the α(1→2) fucose branched in the trisaccharide 2′FL but failed to remove more than 6% of the same fucosyl linkage in the LNFP I. The enzyme is also able to cleave the α(1→4) linkage as observed with the fucosylated pentasaccharide LNFP II but not the α(1→3) linkage of the analog pentasaccharide LNFP III (7.8%). Low linkage specificity has been ascribed for other fucosidases from mollusks, bacteria, and mammals, all of which are active on synthetic substrates like *p*-nitrophenyl–α-L-fucopyranoside or methyl-umbelliferyl α-L-fucopyranoside. Only α-L-fucosidases from almond (Ogata-Arakawa *et al.*, 1977; Scudder *et al.*, 1990) and from the microorganisms *C. perfringens* (Aminoff and Furukawa, 1970), *Xanthomonas manihotis* (Wong-Madden and Landry,

1995), and *Streptomyces* sp. (Sano *et al.*, 1992) were reported to have linkage specificity. Further studies using other fucosylated oligosaccharides are being performed to ascertain the specificity of the *P. maximus* α-L-fucosidase for the α(1→2) and the α(1→4) linkages.

The *P. maximus* α-L-fucosidase catalyzes the hydrolysis of the glycosidic bond of fucosyl unit linked not only to a chromogenic marker or to an oligosaccharide but also to the polysaccharide fucoidan. This result implies the unique property of recognition and hydrolysis of the fucose-fucose linkage because fucoidan is an α-L-fucose-based polysaccharide. To date the molecular structure of algal fucoidan is not fully elucidated. However we and others have recently reported that the structural organization of the *A. nodosum* fucoidan is based on an α-(1→3) and (1→4)-linked fucose backbone (Daniel *et al.*, 1999; Chevolot *et al.*, 1999), unlike to the *F. vesiculosus* fucoidan, which is based on an α(1→3) fucose backbone (Patankar *et al.*, 1993). This structural difference in the fucosyl linkage may explain the difference of the α-L-fucosidase activity toward these two fucoidans. The lack of fucose release from the *F. vesiculosus* fucoidan as well as from the pentasaccharide LNFP III shows the inability of the *P. maximus* α-L-fucosidase to hydrolyze the α(1→3) fucosyl linkage. Furthermore, the enhancement of the fucose hydrolysis from *A. nodosum* fucoidan on addition of sulfatase clearly shows that sulfate groups prevent α-L-fucosidase activity. The *P. maximus* α-L-fucosidase is thus the first enzyme reported to only cleave efficiently the α(1→4)-L-fucose glycosidic bond in a high-molecular-weight polysaccharide. The release of fucose without extensive depolymerization of fucoidan suggests that the hydrolysis of fucose occurs at the nonreducing end of the polysaccharide either at branch or terminal positions.

Enzymes are very useful for the structural analysis of glycans and polysaccharides and for establishing structure–activity relationships in biological systems. The *P. maximus* α-L-fucosidase might be a valuable enzymatic tool to study the structural properties of fucoidan and to improve our knowledge of its biological properties. Recently it has been reported that the fucose branch of the fucoidan could contribute to its biological activities (Patankar *et al.*, 1993; Pereira *et al.*, 1999). This α-L-fucosidase is a unique tool to produce fucoidan devoid of such branches (at least the α1→4 branch) and to assess their biological importance. The action of this exo-glycosidase could be completed by other kind of hydrolases (e.g., of the endo type) that could be isolated from *P. maximus* digestive glands. These additional enzymes, theoretically necessary for fucoidan depolymerization, are currently being sought. Isolation and characterization of such enzymes may enable us to implement a process for the sequential enzymatic hydrolysis of the fucoidan.

Materials and methods

Reagents

The digestive glands of the marine mollusk *P. maximus* were kindly provided by P. Roy (IFREMER, France). Fucoidan from the brown algae *A. nodosum* (M_r 13,000, fucose 42 g/100 g, sulfate 31 g/100 g, uronic acid 5 g/100 g) was extracted and purified as described (Mabeau *et al.*, 1990; Colliec *et al.*, 1991). Fucoidan from *F. vesiculosus* was from Sigma. The sulfatase was prepared as described elsewhere (Daniel *et al.*, 2001). 1-Deoxynojirimycin, 1-deoxygalactonojirimycin, 1-DMJ, fucosylamine, and the human milk oligosaccharides 2′FL, LNFP I, LNFP II, and LNFIII were from Sigma. 1-Deoxyfuconojirimycin was purchased from Calbiochem (France). *p*-Nitrophenyl glycosides (α-L-fucopyranoside, β-L-fucopyranoside, β-D-fucopyranoside, α-D-galactopyranoside, β-D-galactopyranoside, β-D-galacturonide, α-D-glucopyranoside, β-D-glucopyranoside, β-D-glucuronide, α-D-manopyranoside, α-D-manopyranoside, β-D-manopyranoside, α-L-rhamnopyranoside, α-D-xylopyranoside, β-D-xylopyranoside, α-L-arabinopyranoside and β-L-arabinopyranoside) used to assay the enzyme specificity were purchased from Sigma. 6-*N*-Acetylamino-DMJ (6-*N*-Acetylamino-1,5-imino-1,5,6-trideoxy-D-mannitol, Figure 1, compound **1**) was prepared as previously described (McCort *et al.*, 2000). Sodium hydroxide 99.99% used for PAD was from Aldrich. N-Glycosidase F was from Boehringer. Other chemicals and buffer reagents were obtained from commercial sources and were of analytical grade.

Enzyme assays

Glycosidase activity on *p*-nitrophenyl glycosides was determined in 0.1 M acetate buffer, pH 4, containing 0.2 M NaCl and 3.5 mM synthetic substrates at 60°C for 10 min. The *p*-nitrophenol released was measured spectrophotometrically at 405 nm (ε = 17,919 at pH 9.5) in 100-µl aliquots of the reaction solution diluted in 900 µl of 0.1 M borax buffer, pH 9.5 (John, 1995). One unit of α-L-fucosidase activity measured on *p*-nitrophenyl–α-L-fucose is defined as the amount of enzyme required to release 1 µmole of *p*-nitrophenol per min at 60°C and pH 4.0. Fucosidase activity on human milk oligosaccharides was assayed with 2′FL, LNFP I, LNFP II, and LNFP III prepared at 1 mM in 0.1 M acetate buffer, pH 4.0, and incubated at 60°C for 24 h with 0.025 U purified fucosidase. Fucosidase activity on fucoidan was performed by incubating 60 mg of fucoidan with 0.5 U purified fucosidase in 5 ml of 50 mM acetate buffer, pH 5, at 40°C for 5 days. Monosaccharides formed on the enzymatic reaction with the human milk. Oligosaccharides and fucoidan were analyzed by HPLC on an amino column with PAD (see following methods). Samples from the fucoidan reaction were purified by SEC on a PD 10 column (Pharmacia Biotech) eluted with water prior to HPLC analysis to remove high-molecular-weight molecules from samples.

Sulfoesterase activity was routinely assayed with *p*-nitrocatechol sulfate as substrate using the spectrophotometric determination of *p*-nitrocatechol at 515 nm (ε_{515} = 12,200 M^{-1} cm^{-1}) (John, 1995). The reaction was initiated by the addition of sulfatase in 0.5 ml of 0.1 M acetate buffer, pH 4.5, containing 10 mM substrate. The reaction was carried out at 50°C for 10 min, then 750 µl of 1 M NaOH were added to stop the reaction and to reveal the absorbance at 515 nm. Enzyme specific activity was defined as the amount of µmoles of *p*-nitrocatechol released per min per mg protein.

Analytical procedures

Hplc analysis of monosaccharides produced during the enzymatic reaction was carried out on an Astec amino column (25 × 0.46 cm) fitted with a guard column (1 × 0.46 cm) (Interchim, France), and with a pulsed amperometric detector Coulochem 2 (Eurosep, France) according to the following pulse potentials:

E1 = 0.2 V (700 ms, acquisition delay = 650 ms), E2 = 0.7 V (100 ms), E3 = –0.6 V (100 ms). Elution was performed at the flow rate of 0.8 ml · min^{-1} with 0.1 µm filtered and degassed acetonitrile:water (4:1) mobile phase. A postcolumn delivery system (Dionex, France) added under constant pressure (30 psi) a 0.3 M NaOH solution to the column effluent prior to detection.

High-performance SEC analysis of fucoidan was performed using a Zorbax GF-450 (25 × 0.46 cm) linked to a TSK G2000 SW_{XL} column (30 × 0.78 cm), both equipped with a guard column and connected to a Gilson 132 RI refractometer. The column was eluted with 0.05 M phosphate buffer, 0.15 M NaCl, pH 7.3, at a flow rate of 0.5 ml · min^{-1}.

Protein concentration was determined using the Bradford assay system (Biorad) with bovine serum albumin as a standard (Bradford, 1976). Native molecular weight of the α-L-fucosidase was determined by gel filtration chromatography performed on a Superose 12 h column (pharmacia biotech), which was equilibrated and eluted with 0.1 m acetate buffer, ph 5.5, and calibrated with the following molecular weight markers: ferritin (M_r 450,000), α-amylase (M_r 200,000), alcohol dehydrogenase (M_r 150,000), albumin (M_r 67,000), ovalbumin (M_r 43,000), and lactalbumin (M_r 14,200) (pharmacia biotech). Purified α-L-fucosidase was analyzed by 12.5% sds–PAGE after heat denaturation in β-mercaptoethanol according to the method of Laemmli (1970). Protein bands were detected with coomassie blue staining. Glycoprotein staining on polyacrylamide gels was performed with the schiff's reagent (Sigma) after fixation in acetic acid:methanol:water (10:35:55), oxidation by 30 mm periodate in 5% acetic acid (v/v) solution, and neutralization by 10 mm meta-bisulfite in 5% acetic acid (v/v) solution.

Preparation of the 6-amino-DMJ affinity gel

6-*N*-Amino-DMJ (6-amino-1,5-imino-1,5,6-trideoxy-D-mannitol, as the TFA salt, Figure 1, compound **2**) was synthesized from 2-*O*-allyl-6-[(*tert*-butyloxycarbonyl)amino]-3,4-di-*O*-benzyl-1,5-[*tert*-butyloxycarbonyl) imino]-1,5,6-trideoxy-D-mannitol as previously reported (McCort *et al.*, 2000) with slight modifications. Specifically, the hydroxyl groups were first deprotected by sodium in liquid ammonia followed by deprotection of the amine functions. This compound was coupled to NHS-activated Sepharose 4 Fast Flow (Pharmacia Biotech) following the manufacturer instructions. Briefly, after several washes with 1 mM HCl at 4°C, the medium was equilibrated with the coupling buffer (0.2 M $NaHCO_3$, pH 7.9). Two milliliters of 60 mM 6-amino-DMJ trifluoroacetate were then mixed with 4 ml of medium in the same buffer, and the coupling reaction was performed at 4°C overnight. The unreacted groups were blocked by standing the medium in 0.2 M Tris buffer, pH 8, at room temperature for a few hours. After repeated wash cycles with 0.2 M acetate buffer, pH 4, and 0.2 M Tris buffer, pH 8, the resulting coupled gel was stored in 50 mM acetate buffer, pH 5, containing 20% ethanol (v/v).

Purification of P. maximus *α-L-fucosidase*

The digestive glands from the marine mollusks *P. maximus* were homogenized with a Warring blender for 10 min in two volumes of extraction buffer (50 mM acetate, pH 5.5, 0.1 M KCl containing 12 µM of phenylmethylsulfonyl fluoride and 10 µM of leupeptin). After 1 h at 4°C under mild agitation, the homogenate was centrifuged (9000 × *g*) for 20 min. The extract was brought to 30% saturation with $(NH_4)_2SO_4$, stirred for 45 min, and then centrifuged (10,000 × *g*) for 25 min. The supernatant was recovered and the concentration of $(NH_4)_2SO_4$ was gradually increased to 70%. After centrifugation (10,000 × *g*, 25 min), the recovered precipitate was dissolved in 50 mM acetate buffer, pH 5.5, and dialyzed three times against the same buffer. The dialyzed extract was then washed with 30% (v/v) ethyl acetate to remove lipids. After centrifugation (2,000 × *g*, 20 min), the aqueous phase was recovered and dialyzed against 25 mM acetate buffer, pH 5.25, prior the first chromatographic step.

Strong cation exchange chromatography. The dialyzed material was loaded on an SP-Sepharose Fast Flow column (12 × 2.5 cm) (Pharmacia Biotech) which was equilibrated with 25 mM sodium acetate, pH 5.25, at 1 ml · min^{-1}. Protein elution was monitored at 280 nm using a 111B UV detector (Gilson). The column was washed with two bed volumes of 25 mM sodium acetate, pH 5.25, then elution was performed with two column volumes of a linear gradient of NaCl (0 to 0.5 M) in the same buffer (flow rate, 1 ml · min^{-1}). Fractions containing α-L-fucosidase activity measured on *p*-nitrophenyl–α-L-fucose were pooled, diafiltered with 50 mM acetate buffer, pH 7, 0.2 M NaCl and concentrated using an ultrafiltration cell (Amicon) with a 5000-Da membrane.

Immobilized zinc affinity chromatography. The concentrated enzyme solution was applied at a flow rate of 1 ml min^{-1} to a Chelating Sepharose Fast Flow column (9.5 × 1.6 cm) (Pharmacia Biotech) loaded with Zn^{2+} and equilibrated with 50 mM acetate buffer, pH 7, 0.2 M NaCl. Protein elution was followed at 280 nm. The column was washed with two bed volumes of the same acetate buffer, then the fucosidase was eluted by 10 mM imidazole in the acetate buffer. Active fractions were pooled, diafiltrated with 50 mM acetate buffer, pH 5, and concentrated to 5 ml using Ultrafree centrifugal filter device 5K (Millipore).

6-Amino-DMJ affinity chromatography. Routinely, an amount of enzyme extract corresponding to 1 U fucosidase activity was applied onto the 6-amino-DMJ Sepharose 4 Fast Flow column (9.5 × 0.4 cm) equilibrated with 50 mM acetate buffer, pH 5. The column was washed with the same buffer until the absorbance of the eluate (measured at 280 nm) had returned to the baseline. The α-L-fucosidase was then eluted by 50 mM fucose in the same acetate buffer. Fucose was removed from the fucosidase solution by diafiltration with Ultrafree centrifugal filter device 5K, and the enzyme was finally concentrated. The α-L-fucosidase was stored at –80°C in acetate buffer 50 mM (pH 5), with no loss of activity observed after several months of storage.

NMR spectroscopy experiments

NMR spectroscopy was performed on a Bruker DMX 500 spectrometer, operating at the proton Larmor frequency of 500.11 MHz. The experiments were performed with a 5-mm probe equipped with self-shielded Z-gradients. Spectra were recorded at 25°C without suppression of HOD signal. Chemical shifts are reported in ppm using sodium 3-trimethylsilyl-propanoate as internal reference. One-dimensional spectra were acquired over 32K data points using a spectral width of 5000 Hz.

Mutarotation kinetics of α-L-fucose was followed by recording ^{1}H NMR spectra every 2 min for 2 h, after dissolution of 6.1 µmole α-L-fucose in 0.5 ml deuterated 0.1 M acetate buffer, pH 5.5. The ratio between the α- and the β-anomer was calculated via relative integration of H-1-α/H-1-β for H-1 signals at 5.20 and 4.55 ppm, respectively, or for H-6-α/H-6-β methyl signals at 1.21 and 1.25 ppm, respectively. The sample of *p*-nitrophenyl–α-L-fucose was exchanged twice with 99.8% D_2O (Sigma) with intermediate lyophilization, and 5.6 µmole was dissolved in 0.5 ml of the previous deuterated acetate buffer. Purified α-L-fucosidase was obtained as a lyophilized powder after a double exchange in D_2O and was dissolved in 15 µl of the same deuterated buffer. After recording of the spectrum of the substrate, 15 µl of enzyme solution was added to the NMR tube, which was immediately placed back in the spectrometer. The first spectrum was recorded 2 min after the addition of the enzyme, and then spectra were recorded every 2 min for 4 h.

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Abbreviations

2′-FL, 2′-fucosyllactose; DMJ, deoxymannojirimycin; EDTA, ethylenediamine tetra-acetic acid; HPLC, high-performance liquid chromatography; LNFP, lacto-N-fucopentaose; NMR, nuclear magnetic resonance; PAD, pulsed amperometric detection; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; SEC, size exclusion chromatography.

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