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Action of α-L-fucoside from *Octopus vulgaris* hepatopancreas on phospholipid vesicles containing the fucosylated ganglioside FucGM1

A. GIULIANI[1]*, P. PALESTINI[1], A. D'ANIELLO[2] and M. MASSERINI[1]

[1] *Department of Biological Chemistry, the Medical School, University of Milan, Via Saldini, 20133 Milan, Italy*

[2] *Stazione Zoologica, Naples, Italy*

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The behaviour of a highly purified α-L-fucosidase (E.C. 3.2.1.51) extracted from octopus hepatopancreas was studied with phospholipid vesicles composed of phosphatidylcholine (PC) and phosphatidylserine (PS) containing the fucosylated ganglioside FucGM1, a potential natural substrate of the enzyme. The substrate recognition and hydrolysis take place only with PS/FucGM1 mixtures via an association process of the enzyme with the vesicles at acidic pH; the enzyme rapidly and stably binds to PS vesicles but not to PC vesicles. The data suggest that only the PS-associated enzyme is able to hydrolyse FucGM1 embedded in the same bilayer. The enzyme association with FucGM1/PS vesicles is a prerequisite for ganglioside hydrolysis but is followed by irreversible enzyme inactivation.

Keywords: α-L-fucosidase; fucosylated ganglioside FucGM1; interaction with phospholipid vesicles

Introduction

It is commonly accepted that in normal tissues an α-L-fucosidase is required to metabolize fuco-glycoconjugates, including fucogangliosides [1]. Several α-L-fucosidases extracted from different tissues of various animal species have been characterized on artificial substrates and on some natural fuco-oligosaccharides and fuco-glycopeptides [2]; very little is known about α-L-fucosidase activity on fucolipids. Using pig brain we obtained a highly purified fucose-containing ganglioside, FucGM1 [3, 4] and utilized it as the substrate for α-L-fucosidase.

The behaviour of a highly purified α-L-fucosidase from octopus hepatopancreas on FucGM1 ganglioside in micellar form has been investigated in a previous work [5]. In the present study the investigation is extended to FucGM1 embedded in a model membrane system, represented by phospholipid unilamellar vesicles.

In order to study in more detail the interaction of the enzyme with a phospholipidic bilayer, we also investigated the behaviour of α-L-fucosidase in the presence of vesicles containing GM1 ganglioside, which is not a substrate for the enzyme.

* To whom correspondence should be addressed.

Materials and methods

Chemicals and other products

4-Methyl-umbelliferyl-α-L-fucoside (MUB-fucoside) and *p*-nitrophenyl-α-L-fucoside (pNP-fucoside) were obtained from Koch-Light (Colnbrook, Bucks, UK); Ultrogel ACA 34 from LKB (Stockholm, Sweden); and silica gel precoated high performance thin-layer plates (HPTLC, Kieselgel 60, 250 μm thick, 10 × 10 cm) from Merck GmbH (Darmstadt, Germany).

Ganglioside GM1 was extracted from bovine brain and FucGM1 from pig brain, as described by Tettamanti *et al.* [6]. Their identification, structural analysis and purity were assessed as described by Sonnino *et al.* [7]. The final purity of all gangliosides was over 99%, as detected by HPTLC with increasing amounts of ganglioside under the following conditions: solvent system, propan-1-ol:32% (by vol) NH_3:water, 6:2:1, by vol; 2 h run at 20 °C. The spots were visualized by treating with an Ehrlich spray reagent followed by densitometric scanning of the plate. FucGM1 and GM1 were tritium-labelled in the C-3 position of the sphingosine moiety using the method of Ghidoni *et al.* [8] and purified as described by Gazzotti *et al.* [9]. The radiochemical purity was greater than 99% for both compounds, and the specific radioactivity was 0.7

Ci $mmol^{-1}$ and 1.0 Ci $mmol^{-1}$ for FucGM1 and GM1 respectively.

Egg phosphatidylcholine (egg PC), grade 1, and bovine spinal cord phosphatidylserine (bovine PS), grade 1, were purchased from BDH, Poole, England.

Preparation of α-L-fucosidase

α-L-Fucosidase (EC 3.2.1.51) from octopus hepatopancreas was prepared as described by D'Aniello *et al.* [10]. The specific activity of the purified enzyme assayed on pNP-fucoside, as described by D'Aniello *et al.* [10] and on MUB-fucoside, as described by Masserini *et al.* [5], was 38.9 and 23 μmol min^{-1} per mg protein, respectively.

Preparation of phospholipid vesicles

Small unilamellar vesicles composed of pure phosphatidylcholine or phosphatidylserine, either with or without 10% (molar) FucGM1 or GM1 were prepared as follows: the lipids were dried using a chloroform/methanol solution under an N_2 flow, lyophilized and resuspended in 50 mM KCl to a lipid concentration of 10 mM: small unilamellar vesicles from this suspension were obtained by sonication and centrifugation following the method of Barenholz *et al.* [11], modified as described by Masserini *et al.* [12]. Phospholipid phosphorus was determined in the final vesicle preparation by the method of Bartlett [13] and brought to a final lipid concentration of 7 mM with a citrate-phosphate buffer, 50 mM, pH 3.4. In all ganglioside-containing vesicle preparations a small amount (10^6 dpm ml^{-1}) of ^{3}H-labelled ganglioside was also present.

All the experimental data refer to samples of 50 μl final volume containing a vesicle preparation with a total lipid concentration of 7 mM.

Assay of α-L-fucosidase activity on MUB-fucoside after preincubation with vesicles

Vesicle suspension (50 μl) was preincubated at 37 °C with different amounts of enzyme preparation (3–60 μg), for varying times (5–60 min) after which activity was assayed on MUB-fucoside. For this purpose, 5 μl aliquots of the pre-incubation mixtures were taken at different incubation times and immediately transferred to a test tube containing 250 μl of 0.25 mM MUB-fucoside in 25 mM citric acid–sodium phosphate buffer, pH 5.6 and incubated for 2 min at 37 °C, as previously described [5]. The reaction was stopped by the addition of 3 ml of a 0.2 M glycine/NaOH buffer, pH 10.5 and the fluorescence immediately measured with a Jasco FP 770 spectrofluorometer.

The activities measured on MUB-fucoside at the various incubation times were expressed as the percentage residual activity, taking as 100% the activity at zero time.

Formation of enzyme/phospholipid vesicle complexes

The method previously described [5] for detection of fucosidase/FucGM1 complexes was followed with minor modifications: the incubation mixtures containing PC or PS vesicles and α-L-fucosidase were processed by column chromatography (25 × 10) on Ultrogel AcA34 equilibrated with a 50 mM citrate-phosphate buffer, pH 3.4. As previously demonstrated [5] this resin is able to retain the free enzyme completely. The incubation mixtures (50 μl) were applied to the column and eluted with the same buffer at a flow rate of 5 ml h^{-1}; 1 ml fractions were automatically collected. The elution of phospholipid vesicles was monitored by recording the turbidity (at 450 nm) and by phosphorous assay followed by radioactivity counting of the radiolabelled ganglioside, when present. Phospholipid-containing fractions were checked for protein content.

Determination of α-L-fucosidase activity on FucGM1 inserted in phospholipid vesicles

The vesicle suspension (50 μl) at pH 3.4 was incubated at 37 °C with different amounts of enzyme preparation (3–60 μg), for varying times (5–60 min).

The incubations were stopped at different times by the addition of four volumes of tetrahydrofuran. The gangliosides, extracted by the method of Tettamanti *et al.* [6], were submitted to HPTLC on silica gel plates as described above. Their identification was done by co-chromatography with pure standard GM1 and FucGM1 (unlabelled and radiolabelled). Residual FucGM1 and released GM1 were quantified by radiochromatoscanning with a Berthold TLC Linear analyser model LB 282, equipped with an Apple II Europlus computer, as previously described [5]. The extent of hydrolysis has been expressed as the relative percentage of FucGM1 hydrolysed = (released GM1/(released GM1 + residual FucGM1) × 100.

Fluorescence spectroscopy experiments

The tryptophan fluorescence spectrum of the enzyme was determined for the different incubation mixtures. The emission spectrum was recorded every 3 min up to 2 h using an exciting wavelength of 282 nm, in a Jasco spectrofluorometer mod.FP 770.

The static quenching [14] due to acrylamide addition was evaluated by measuring the tryptophan fluorescence intensity before (F_0) and after (F) the addition of appropriate amounts of a 7 M acrylamide solution, to give from 0.1 M to 0.7 M final concentration of acrylamide, using an exciting wavelength of 282 nm and an emission wavelength of 337 nm.

Other analytical methods

The protein content was determined by the method of Lowry *et al.* [15], with bovine serum albumin as the standard; ganglioside-bound *N*-acetylneuraminic acid was assayed by the method of Svennerholm [16], with *N*-acetylneuraminic acid as the standard; phospholipid phosphorus was determined by the method of Bartlett [13].

Results

Enzymatic activity determination under different experimental conditions

(a) α-L-Fucosidase activity using MUB-fucoside as the substrate

The activity of α-L-fucosidase on MUB-fucoside was measured after different preincubation times with phospholipid vesicles using 3 μg of enzyme protein per sample. As shown in Fig. 1 the preincubation with egg PC vesicles (with or without FucGM1 or GM1) did not affect the activity of α-L-fucosidase on MUB-fucoside. However, when incubated with PS or FucGM1/PS and GM1/PS vesicles, the activity decreased as the incubation time increased. The incubation mixtures of the enzyme with the vesicles were submitted to gel filtration. The vesicle-containing fractions were collected, concentrated by ultrafiltration and checked for protein content. In all cases the percentage of vesicle-associated protein eluted from the column exactly corresponded to the percentage decrease in activity (measured on MUB-fucoside) after incubation. As an example, in a sample where there was an 80% decrease in the activity on MUB-fucoside following incubation, 80% of the protein content was co-eluted with the vesicles. This suggests that the decrease in activity recorded during incubation is due to the association of the enzyme with the vesicles which is similar to the findings previously obtained with micellar gangliosides [5]. Therefore the decrease in activity can be used as a measure of the association of the enzyme with the phospholipid vesicles. Conversely all the incubation mixtures with PC vesicles were eluted without the enzyme protein which was retained by the column.

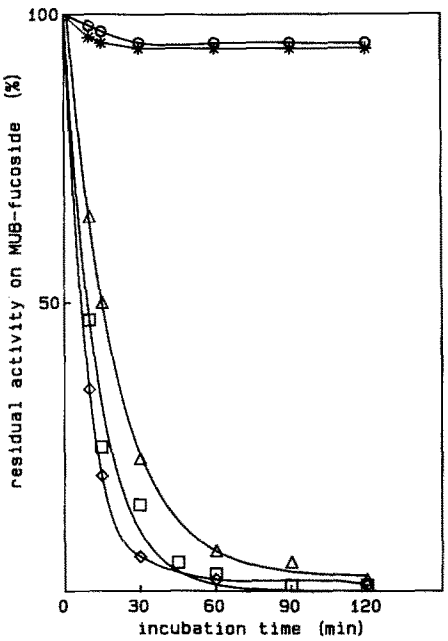


Figure 1. Residual activity of the α-L-fucosidase on MUB-fucoside against incubation time with PC or PS phospholipid vesicles in the presence or absence of FucGM1 (or GM1) ganglioside (see text for conditions of incubation). ○, enzyme in buffer; △, PS vesicles; □, PS/FucGM1 vesicles; ◇, PS/GM1 vesicles; *, PC and PC/FucGM1 or PC/GM1 vesicles (a single curve is represented since the three samples differ only by the experimental error).

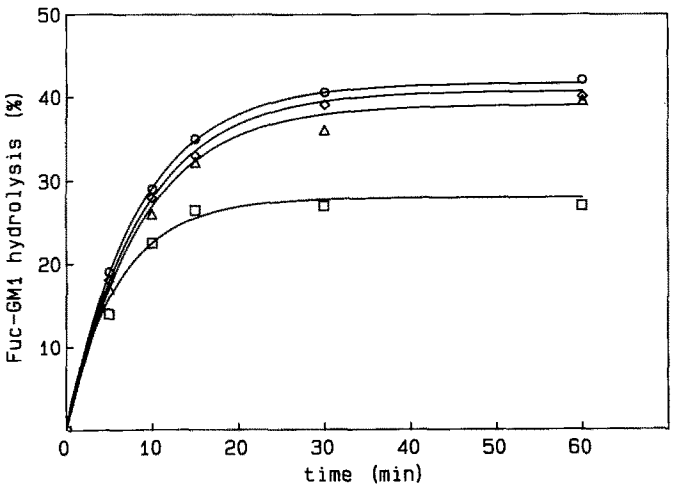
(b) α-L-Fucosidase activity using FucGM1 as the substrate

Using FucGM1/PC unilamellar vesicles no hydrolytic activity on the ganglioside could be detected when using protein concentrations of 3–60 μg per 50 μl. On the contrary, when using FucGM1/PS unilamellar vesicles under the same conditions the α-L-fucosidase was able to cleave FucGM1.

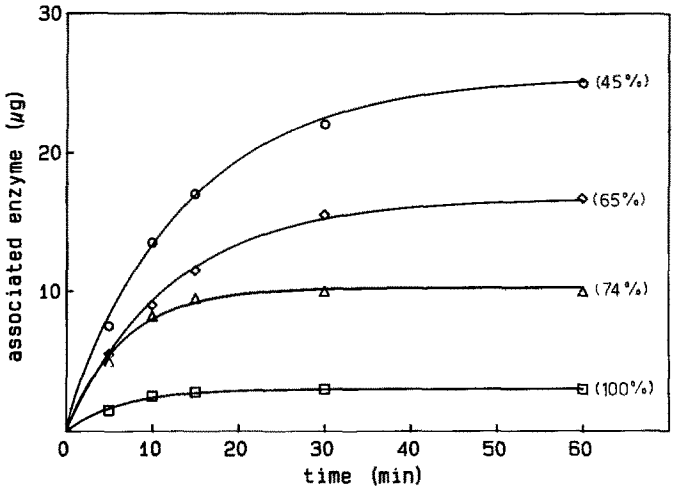
Figure 2a shows the time course of FucGM1 hydrolysis, inserted as 10% molar in PS vesicles (7 mM total lipid concentration), in the presence of different enzyme concentrations. The reaction reached a plateau after 30 min of incubation time. The linearity with incubation time continues for only the first 5 min (not shown). Under the same conditions the amount of enzyme bound to the vesicles was evaluated both by gel-filtration and by assay of the residual activity on MUB-fucoside. The results are shown in Fig. 2b: the amount of enzyme protein bound to the vesicles increased on increasing the amount of protein in the incubation mixture: however, complete (100%) enzyme association with the vesicles was found only at an enzyme concentration of 3 μg per 50 μl.

In order to establish whether the free or the bound enzyme is the form acting on the ganglioside substrate, the rate of FucGM1 hydrolysis was plotted against the amount of either bound or free enzyme. The relationship between the extent of FucGM1 hydrolysis and the amount of enzyme bound to the vesicles is linear only in the initial part of the curve – for enzyme concentrations up to 3 μg per 50 μl (Fig. 3). In this range of enzyme concentration, all the enzyme present in the incubation mixture, after 15 min of incubation, is coeluted with phospholipids from the chromatography column, indicating its complete association with the vesicles. On the other hand, the amount of enzyme which is not associated with the vesicles (free enzyme) is inversely related to the extent of FucGM1 hydrolysis (data not shown). This indicates that the free form of the enzyme cannot be catalytically active on the ganglioside substrate.

Under experimental conditions where the relationship between the extent of fucoganglioside hydrolysis and the enzyme protein or the incubation time was linear, the dependence of the rate of substrate hydrolysis on the substrate concentration (V/S) has been evaluated. On varying the substrate concentration by using increasing amounts of the FucGM1/PS vesicle preparation, a classical Michaelis-Menten kinetics was obtained, from which an apparent V_{max} of 22.1 nmol FucGM1 hydrolyzed per min per mg protein and a K_m of 0.24 mM FucGM1 were calculated (Fig. 4).



(a)



(b)

Figure 2. Behaviour of α-L-fucosidase against FucGM1 (10% molar)-PS vesicles (7 mM total lipid concentration, pH 3.4): (a) time course of FucGM1 hydrolysis at different enzyme concentrations; (b) time course of enzyme association to FucGM1-PS vesicles under the same conditions (in parentheses the maximal association expressed as a percentage of the total enzyme incubated with the vesicles). □, 3 μg protein per 50 μl; △, 15 μg protein per 50 μl; ◇, 30 μg protein per 50 μl; ○, 60 μg protein per 50 μl.

Fluorescence experiments

The tryptophan fluorescence intensity of the enzyme was measured in the presence of increasing acrylamide concentration, before and after preincubation with FucGM1/PS vesicles, in the conditions giving the maximum association (3 μg of enzyme in 50 μl of 7 mM phospholipid vesicles). Acrylamide in a given concentration causes quenching of tryptophan fluorescence (shown by the increase in F_0/F ratio) when added to the enzyme solution. When the enzyme is preincubated with PS/FucGM1 vesicles, the increase in F_0/F ratio is smaller at the same acrylamide concentration, suggesting that the accessibility of acrylamide to the tryptophan residues of α-L-fucosidase is diminished (Fig. 5). We also observed under the same conditions a blue shift in fluorescence maximum of the tryptophan spectrum from a starting value of 337 nm to a final value of 327 nm after 30 min of incubation (Fig. 6), corresponding to the time required for the complete association of the enzyme with the vesicles.

Under the conditions where some of the enzyme was free and some associated with the vesicles, two peaks in the tryptophan fluorescence emission spectrum were present, one at 337 nm and the second at 327 nm; the area of this latter is proportional to the extent of FucGM1 hydrolysis

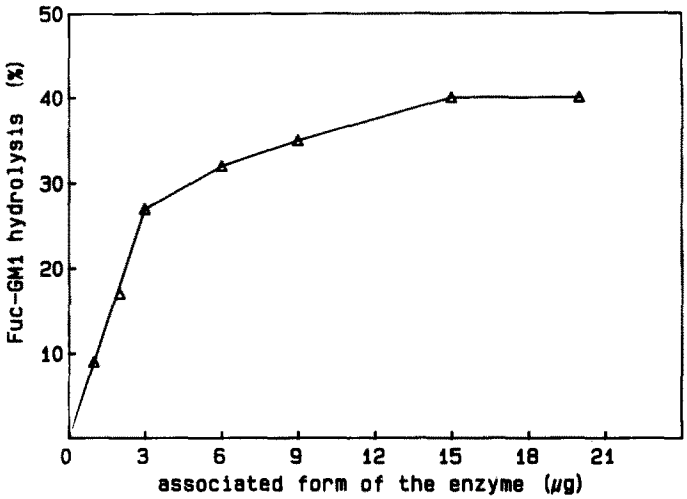


Figure 3. Dependence of the extent of FucGM1 hydrolysis on the amount of α-L-fucosidase associated with FucGM1 (10% molar)-PS vesicles. Experimental conditions: 7 mM total lipid concentration, 15 min incubation time, pH 3.4; 50 μl final volume.

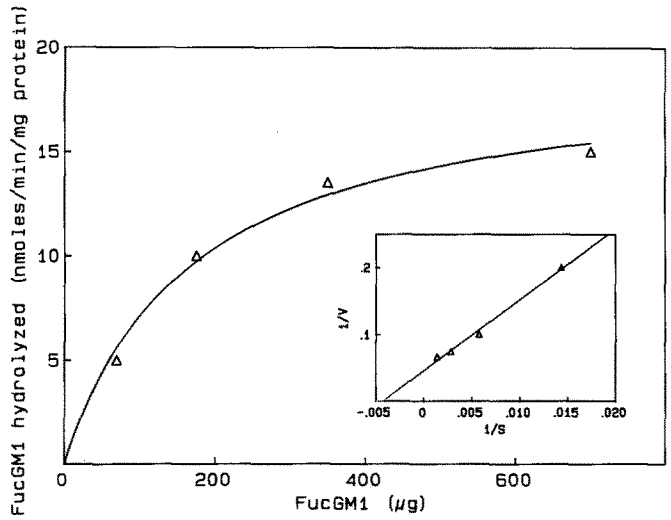


Figure 4. Dependence of the rate of FucGM1 hydrolysis on the ganglioside concentration (V/S relationship) catalysed by α-L-fucosidase on FucGM1 inserted (10% molar) in PS vesicles. Experimental conditions: 3 μg of protein per 50 μl, 5 min incubation time, pH 3.4. The reciprocal 1/V vs 1/S plot shown in the inset.

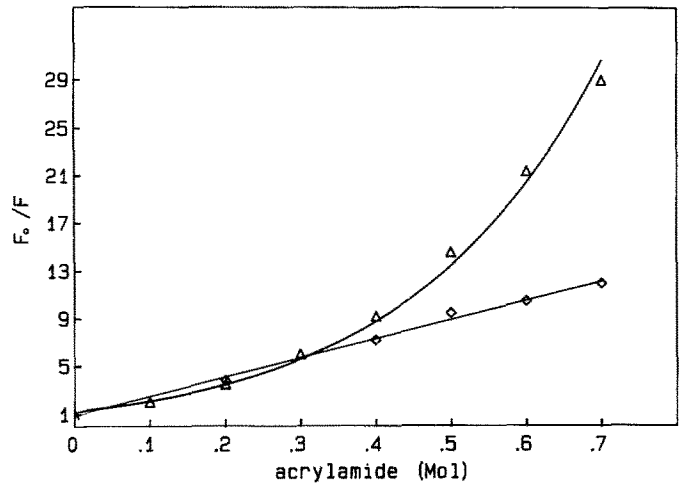


Figure 5. α-L-Fucosidase tryptophan fluorescence quenching with acrylamide. Experimental conditions: 3 μg enzyme as protein, 7 mM total lipid concentration, 30 min incubation time, 50 μl final volume): △, in the absence of FucGM1-PS vesicles; ◇, after incubation with FucGM1-PS vesicles. F_0 tryptophan fluorescence in the absence of acrylamide; F tryptophan fluorescence in the presence of acrylamide.

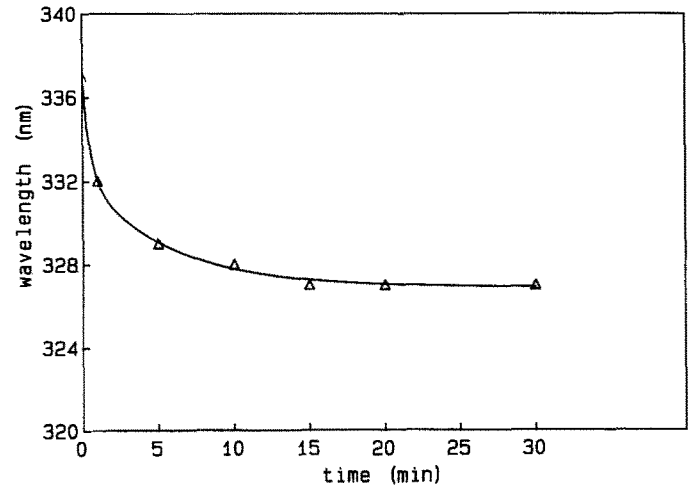


Figure 6. Blue-shift of the fluorescence maximum of the tryptophan spectrum as the function of incubation time with FucGM1-PS vesicles.

rather than to the total amount of enzyme bound to the vesicles, as shown by the linear relationship, up to 40% of hydrolysis, shown in Fig. 7. In other words almost two types of association of the enzyme with the vesicles must take place and the blue-shifted peak is probably linked only to the portion of bound enzyme that is catalytically active.

Discussion

The behaviour of octopus α-L-fucosidase proves an example of the complex nature of the recognition process between a soluble enzyme and a glycolipid substrate: the catalytic activity varies dramatically depending on the physico-

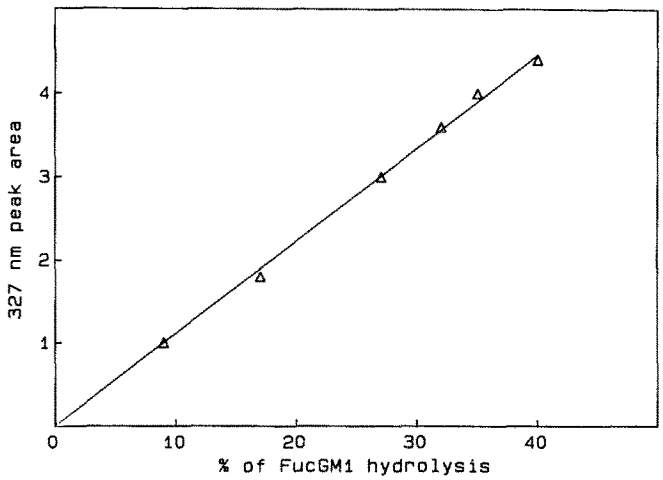


Figure 7. Area of the 327 nm peak as the function of the percentage of FucGM1 hydrolysis.

chemical features of the substrate itself. In fact, while FucGM1 in micellar form is a good substrate for the free form of the enzyme [5], the same fucoganglioside embedded in PS vesicles can be hydrolysed only after association of the enzyme with the vesicle itself. At acidic pH the enzyme forms a stable association only with phosphatidylserine vesicles, losing its activity on the artificial substrate MUB-fucoside, but meanwhile acquiring the ability to hydrolyse FucGM1 present in the bilayer. Under the same experimental conditions the enzyme does not associate with the phosphatidylcholine vesicles, fully maintaining its activity towards MUB-fucoside, and is not able to hydrolyse PC-bearing FucGM1. This opposing behaviour of PC and PS vesicles with respect to the enzyme association suggests that the surface charge of the liposomes where FucGM1 is inserted could play an important role in the interaction with the enzyme. The evaluation of this hypothesis, which could be tested using other acidic lipids such as phosphatidylglycerol, phosphatidic acid and sulfatides, deserves further experimental work and is currently in progress in our laboratories.

Only part of the PS-associated enzyme is able to cleave FucGM1, as shown by the tryptophan fluorescence experiments, suggesting the existence of two different pools of vesicle-bound enzyme: the first one capable of hydrolysing FucGM1 and the second one catalytically inactive towards the ganglioside substrate. The glycolipid behaves like a suicide-substrate [17], since every experimental condition where FucGM1 hydrolysis is detected, eventually results in the formation of a stable and catalytically inactive enzyme complex.

This behaviour is likely to be extended to other fucosidases; the preliminary results published by Hopfer *et al.* [18] on two α-L-fucosidases from human liver and human brain, where FucGM1 is present at the membrane level, have also shown a non-linear relationship of the fucose released with the incubation time. Moreover, the same

authors have ascertained that neither of these human fucosidases requires an activator protein to express their catalytic activity on FucGM1. The need for activator proteins in glycolipid catabolism is common to many mammalian lysosomal glucosidases [19, 20], included an α-L-fucosidase extracted and purified from pig brain in our laboratory (unpublished results).

It may be concluded that α-L-fucosidases from octopus hepatopancreas expresses a modulation of its activity depending on the physicochemical form in which the glycolipid substrate, FucGM1, is presented. Thus, maximal hydrolysis is obtained when the substrate is present in micellar form [4], partial hydrolysis when the substrate is present in a PS bilayer, and lack of hydrolysis when FucGM1 is inserted in a PC bilayer. Whether these interactions are also relevant at the level of a biological membrane where other lipids and proteins are present, remains an open question.

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