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Use of resorufin-labelled N-glycopeptide in a high-performance liquid chromatography assay to monitor endoglycosidase activities during cultivation of *Flavobacterium meningosepticum*

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Peptide-N^4-(*N*-acetyl-β-glucosaminyl) asparagine amidase F (PNGase F) and endo-β-*N*-acetyl glucosaminidase F (Endo F) activities were monitored during cultivation of *Flavobacterium meningosepticum* using a new fluorescence-HPLC procedure based on a commercially available substrate. The PNGase F activity reached a maximum level at the end of the log phase and remained constant during the stationary phase, while Endo F continuously increased until late stationary phase. PNGase F obtained at the end of the log phase was less contaminated by other proteins compared with late stationary phase.

Keywords: Endoglycosidases assay, PNGase F, fluorescence-HPLC

Abbreviations: Con A, concanavalin A; Endo F, endo-β-*N*-acetyl glucosaminidase F (EC 3.2.1.96); GlcNAc, *N*-acetylglucosamine; PNGase F, peptide-N^4-(*N*-acetyl-β-glucosaminyl) asparagine amidase F (EC 3.5.1.52).

Endo-β-N-acetyl glucosaminidase F (Endo F, EC 3.2.1.96) [1] and peptide-N^4-(*N*-acetyl-β-glucosaminyl) asparagine amidase F (PNGase F, EC 3.5.1.52) [2], from *Flavobacterium meningosepticum*, have proven useful tools for the study of both protein and carbohydrate moieties of glycoproteins [3, 4]. Having the broadest substrate specificity of all known endoglycosidases, PNGase F has become the most widely used enzyme for removing N-glycosidically linked carbohydrate chains from glycoproteins. Several assay procedures have been proposed for the detection of those activities, mainly based on the use of either radioactive or dansylated glycopeptide or glycoprotein substrates [5–8], all of them prepared by the authors and not commercially available.

Resorufin-labelled N-glycopeptide (Boehringer Mannheim), a high mannose fluorescent substrate, has been used initially in a colorimetric assay [9]. Recently, the hydrolysis products were analysed by thin layer or paper chromatography [4], allowing their relative quantification. Using the commercially available resorufin-labelled N-glycopeptide in a new, easy and reproducible quantitative HPLC assay procedure, we monitored PNGase F and Endo F activities during cultivation. We looked for maximum PNGase activity in the culture medium, in order to optimize an early purification procedure [5, 6]. We demonstrated that a maximum PNGase activity is obtained at the end of the log phase. This observation is of importance for carrying out subsequent purification steps of PNGase F and also for understanding the synthesis and secretion of the enzymes.

Materials and methods

Bacterial growth and purification of the endoglycosidases

Flavobacterium meningosepticum was obtained from the American Type Culture Collection (ATCC 33958) and grown as described [5] with 1 l of M9 medium supplemented with 0.5% casaminoacids (Difco) in 3 l flasks. Enzyme purification was carried out as described by Tarentino *et al.* [5]. After concentration and ammonium sulfate precipitation, the extract was applied to a TSK HW-55(S) column (2.0 cm × 175 cm) equilibrated in 20 mM Tris-HCl, pH 7.1, containing 100 mM NaCl and 5 mM EDTA (TSK column buffer). The column was developed at 12 ml h^{-1}, and 2 ml fractions were collected.

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Assay procedure

Enzyme preparation (2 µl) was added to 10 µl (400 ng) of resorufin-labelled N-glycopeptide (Boehringer Mannheim, France, Cat. nr. 1016776) in the PNGase buffer (50 mM Tris-HCl, pH 8.0) or the Endo F buffer (50 mM sodium acetate, pH 4.0). After incubation at 37 °C, the reaction was stopped by acidification (pH 2.0 with 5 µl trifluoroacetic acid) and the products were applied to a 5 µm C18 (10 cm cartridge, Brownlee) or to a 3 µm Spherisorb ODS2 (10 cm × 0.46 cm, Colochrom) reversed phase column. Separation was achieved by a linear gradient produced by a Spectra-Physics SP8800 ternary HPLC pump from 0.05% trifluoroacetic acid (solvent A) and acetonitrile containing 0.05% trifluoroacetic acid (solvent B). The elution program was A90%–B10% to A85%–B15% within 10 min followed by a 5 min isocratic elution at the final conditions. The flow rate was 1 ml min^{-1}. The products were detected with a Waters 470 scanning fluorescence detector using excitation and fluorescent emission wavelengths of 467 nm and 559 nm, respectively. A WINner (Spectra-Physics) system was used for data acquisition. The hydrolysis rate was expressed as the quantity of substrate hydrolysed per min (pmol min^{-1}). One unit of PNGase and Endo F was defined according to Tarentino *et al.* [5] as the amount of enzyme required to convert 1 nmol (1939 ng) of FII fraction from resorufin-labelled N-glycopeptide to the final product, resorufin-peptide, in 1 min at 37 °C in 50 mM Tris-HCl, pH 8.0, or 50 mM sodium acetate, pH 4.0, buffer, respectively.

Purification and characterization of the substrate

The substrate, 100 µg in 200 µl, was applied to a 5 µm C18 Nucleosil large pore (30 nm) column (25 × 0.46 cm Colochrom) and fractionated using isocratic elution with A90%–B10% (solvent system described for the assay procedure) for 30 min, followed by a gradient to A40%–B60%. Fractions were pooled, evaporated under vacuum and reconstituted into the PNGase F or Endo F buffer. The carbohydrate portion of fraction FII was characterized after permethylation [10] by fast atom bombardment mass spectrometry (FAB-MS) with a Kratos Concept II HH spectrometer operating in the positive mode (8 keV) and a FAB gun with xenon kinetic energy of 7–8 keV, using thioglycerol–trifluoroacetic acid (1.5%) as the matrix. Amino acid composition was obtained with the PITC method [11].

Results

Assay procedure

The structure of the commercially available substrate as well as the cleavage sites of Endo F and PNGase F are presented in Fig. 1. The heterogeneity of the glycopeptide, described earlier [4], can be due to the variability of the

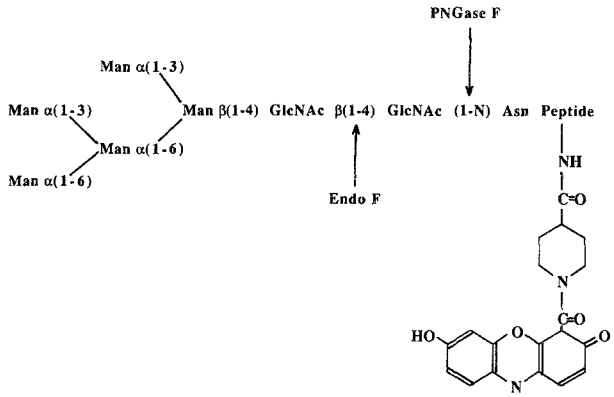

Figure 1. Proposed structure for the oligomannoside-type, resorufin-labelled N-glycopeptide (according to the manufacturer) used as substrate for the assay of endo β-*N*-acetylglucosaminidase F (Endo F) and peptide-N^4-(*N*-acetyl-β-glucosaminyl)asparagine amidase F (PNGase F). Arrows indicates the cleavage sites for Endo F and PNGase F.

glycan and/or the peptide part. In fact, $Man_5GlcNAc_2$-peptide may be contaminated by higher oligomannoside-type glycopeptides, since the substrate was prepared from the 7S soybean glycopeptides [12] by digestion with jack bean α-mannosidase. In addition, the peptide part can be heterogeneous due to the pronase E digestion used during the isolation procedure from the soybean storage proteins [12, 13].

The resorufin-labelled substrate was separated into three fractions (Fig. 2) and each fraction was digested with PNGase F or Endo F. Fraction FIII was not modified (data not shown), suggesting unreacted resorufin or resorufin-labelled peptides. From fraction FI, at least two peptides (PI, PII) or two glycopeptides (GPI, GPII) were obtained (Fig. 3). Only one product was obtained from fraction FII, either for PNGase F (PIII) or endo F (GPIII) action (Fig. 4). FAB-MS analysis of the permethylated FII glycopeptide revealed only characteristic fragments from its sugar part. Ions at *m/z* 1280 correspond to $Man_5GlcNAc$ and at *m/z* 1525 correspond to $Man_5GlcNAc_2$. Amino acid composition was Asp/Ala/Ser/Thr, 1.0/1.04/0.8/0.85,

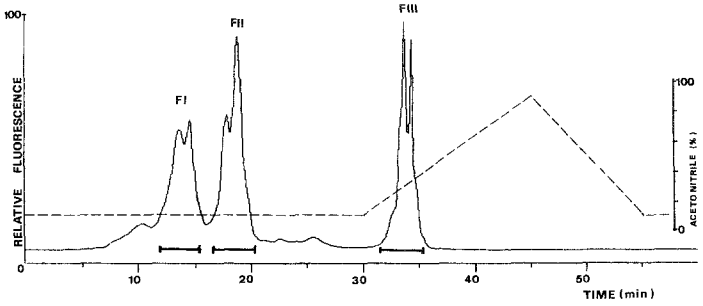

Figure 2. Fractionation of the total substrate by chromatography on a C_{18} reversed phase column. The procedure is described in the Materials and methods section.

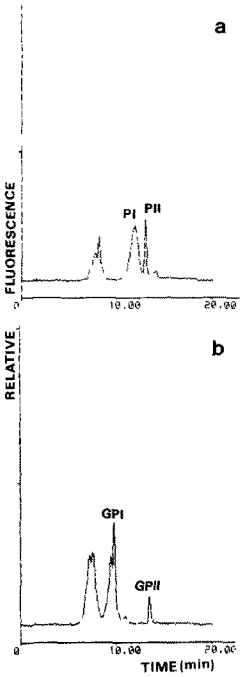

Figure 3. HPLC separations of the hydrolysis products from fraction FI of the resorufin-labelled substrate by the action of PNGase F (a) and Endo F (b). For chromatographic conditions, see the Materials and methods section.

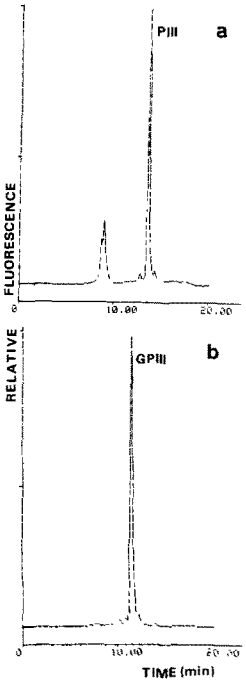

Figure 4. HPLC separations of the hydrolysis products from fraction FII of the resorufin-labelled substrate by the action of PNGase F (a) and Endo F (b). For chromatographic conditions, see the Materials and methods section.

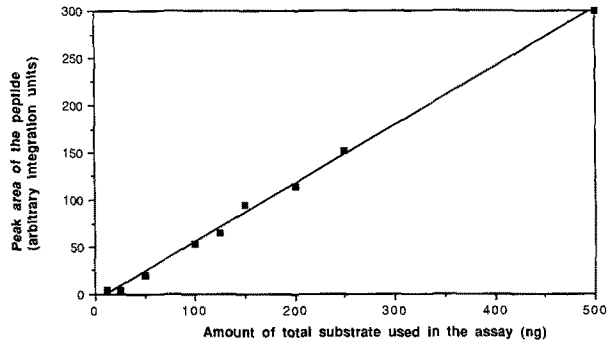

Figure 5. Use of the resorufin-labelled substrate for the quantification of PNGase F activity. Relationship between the area of the peptide (PIII) and the initial concentration of substrate. (Correlation coefficient 0.992.)

demonstrating a tetrapeptide. Thus, the molecular weight of the native glycopeptide is 1939.

The area of the peak corresponding to the PNGase F produced peptide (PIII) was demonstrated to be proportional (correlation coefficient 0.992) to the initial substrate concentration within the range of 20–500 ng (Fig. 5). Amounts below 20 ng, corresponding to the detection threshold must be avoided.

Linearity of the substrate hydrolysis rate over the incubation period was studied in order to choose initial rate conditions for the assay. With the substrate concentration used (400 ng/10 µl), initial rate conditions were obtained with 2 µl of column fraction and up to 5 min of incubation with an excellent correlation coefficient of 0.998 (data not shown). Reproducibility of the method was studied by repeating twelve times the assay with 400 ng and a 2 min incubation period. Standard deviation ($n = 12$) reached only 3%. A good correlation was also observed (correlation coefficient of 0.983) between the hydrolysis rate of the resorufin-labelled substrate and the input of PNGase F (up to 50 munit) in the assay (Fig. 6).

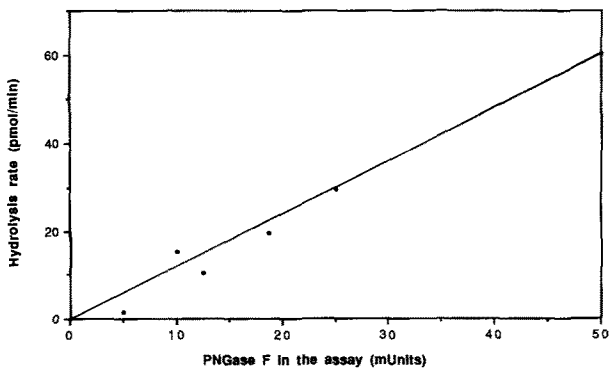

Figure 6. Relationship between hydrolysis rate of 400 ng of the resorufin-labelled substrate and the amount of PNGase F. (Correlation coefficient 0.983.)

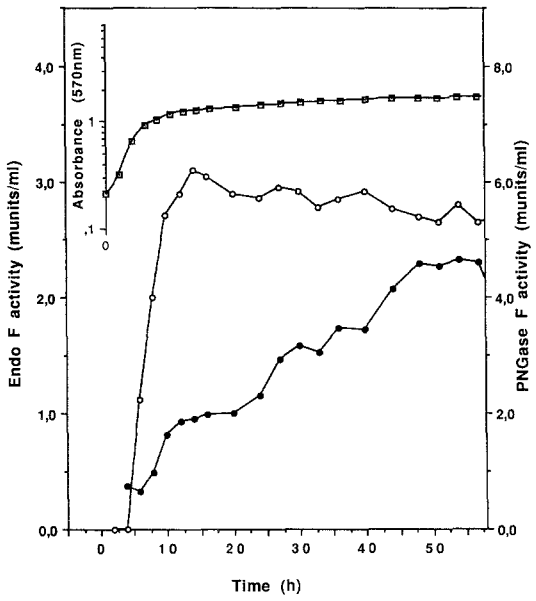

Figure 7. Evolution of PNGase F and Endo F activities during cultivation of *Flavobacterium meningosepticum*. Fractions were assayed for Endo F (●) and PNGase F (○) activities with the resorufin-labelled substrate, as described in the Materials and methods section.

Monitoring PNGase F and Endo F activities during cultivation of Flavobacterium meningosepticum

It was previously reported [5] that PNGase F and Endo F are secreted into the medium during cultivation of *Flavobacterium meningosepticum*, and approach levels of 7 munits ml^{-1} and 3.5 munits ml^{-1}, respectively, late in the stationary phase (44–48 h).

In order to optimize an earlier purification procedure [5, 6], we looked for maximum PNGase activity in the culture medium. The growth curve as well as concomitant evolution of PNGase F and Endo F activities are presented in Fig. 7.

We observed that a maximum PNGase F activity (6 munits ml^{-1}) was obtained at the end of the log phase (10–12 h), while Endo F was at a 1.2 munits ml^{-1} level. PNGase F remained almost constant during the stationary phase (5 munits ml^{-1}) but Endo F continuously increased until late in the stationary phase (2.5 munits ml^{-1}). The PNGase F/Endo F ratio was five at the end of the log phase and tended towards two late in the stationary phase.

We applied the purification process [6] to the culture filtrate obtained at 10–12 h and 44–48 h and compared the quality of these two preparations. After concentration by ultrafiltration and ammonium sulfate precipitation the crude extracts were chromatographed on the TSK HW-55(S) column. With the crude 44–48 h extract, Endo F was recovered in fractions 188–200 (Fig. 8B). PNGase F was retarded on the column and eluted in fractions 198–216. The separation of these two activities was enhanced with the culture filtrate obtained at 10–12 h (Fig. 8A). Indeed,

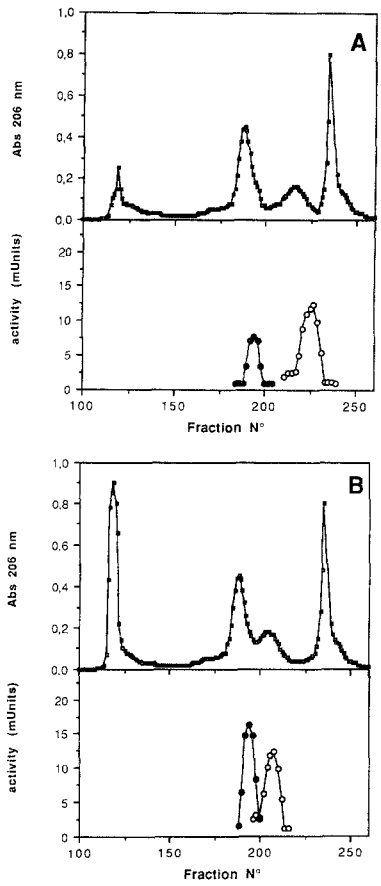

Figure 8. Chromatography of the culture filtrate obtained at 10–12 h (A) and 44–48 h (B) on the TSK HW-55(S) column. Fractions were assayed for Endo F (●) and PNGase F (○) activities with the resorufin-labelled substrate. The chromatographic and assay conditions are described in the Materials and methods section.

while the Endo F activity was recovered in fractions 188–200, PNGase F was particularly retarded and eluted in fractions 220–230.

Discussion

We have designed a new and very sensitive endoglycosidase assay procedure based on the use of a commercially available substrate. Previously, several assay procedures have been used by different authors, using their own substrates, sometimes obtained by tedious purification procedures.

Thus, Nuck *et al.* [7] estimated endoglycosidic activities by measuring the radioactivity of enzymatically released oligosaccharides from intact radiolabelled glycoproteins. In another method, an octaglycopeptide, isolated from turkey ovomucoid after digestion with *Staphylococcus aureus* protease and purification on Sephadex G-50 and DEAE-Sepharose, was used by Mussar *et al.* [8] in an

FPLC assay procedure, the product being detected at 269 nm.

Plummer *et al.* [2] prepared a pentaglycopeptide from fetuin by reduction, alkylation, thermolytic digestion and chromatography on columns of Sephadex G-50, Dowex 50-X2 and DE-52. From ovalbumin, the substrates $Man_5GlcNAc_4Asn$ and $Man_6GlcNAc_4Asn$ have been prepared by Pronase and Carboxypeptidase A digestion and separated into homogeneous fractions by several column fractionation steps [5] or by cyanogen bromide cleavage, Concanavalin A–Sepharose chromatography and tryptic cleavage [14]. In addition to the various isolation and purification steps, these substrates need to be derivatized. This is generally done either by dansylation [5, 6] or dabsylation [15] before using them in batch or HPLC assay procedures.

The commercially available substrate we used, the resorufin-labelled N-glycopeptide from Boehringer, is actually a mixture of several glycopeptides, but they are already labelled, and fractionation of this mixture is easily performed with one step reversed phase high performance liquid chromatography. One of the fractions obtained was demonstrated to give only one peptide (or GlcNAc-peptide) by PNGase (or Endo F) digestion (Fig. 4). This fraction was chosen to perform the assay which is very sensitive since only 400 ng, corresponding to 0.2 nmol, are needed per experimental point.

We noticed that labelling of the substrate greatly enhances the sensitivity of the assay. Thus, the use of dansyl, dabsyl or resorufin label increased the sensitivity 20–400-fold (unlabelled octaglycopeptide, 35–80 nmol per assay [8]; dansyl fetuin pentaglycopeptide, 2 nmol per assay [15]; resorufin labelled glycopeptide, 0.2 nmol per assay). The sensitivity of the labelled glycopeptides is comparable to that of radiolabelled substrates (radiolabelled asialotransferrin, 0.6 nmol per assay [7]). In addition, the HPLC procedures used by us and other authors [8, 15] allow the discrimination of Endo F and PNGase activities.

The same batch substrate preparation could be employed over a large period of time (at least one year) without any noticeable degradation. It is probable that differences occur between different batch preparations, but the HPLC fractionation and the use of fraction FII ensure reliable results.

The good reproducibility of the assay, always carried out under initial rate conditions, and its sensitivity allow experiments such as monitoring of endoglycosidase activities during isolation and purification processes, or studies of the evolution of such activities during cultivation of the producing cells.

In this paper, the assay was succesfully applied in order to study the evolution of PNGase F and Endo F activities during cultivation of *Flavobacterium meningosepticum.*

Production of PNGase F is observed only during the log phase, while for Endo F it continues even during the stationary phase (Fig. 7). In addition, the PNGase F activity expressed per absorbance unit is constant, while an increase is observed for Endo F. This phenomenon probably reflects a different mechanism of gene expression and synthesis or a distinctive secretion process for each enzyme. The profile obtained for Endo F can also be explained by the occurrence of three distinct enzyme species which have been characterized recently [16].

The retardation of PNGase F upon TSK HW-55(S) chromatography is due to the hydrophobic interactions with the gel matrix [5]. These interactions are more pronounced for PNGase F prepared from the 10–12 h than from the 44–48 h cultural filtrate. For the 44–48 h preparation, PNGase F probably interacts with contaminants rather than the gel matrix, and is less retarded. When the 44–48 h preparation is rechromatographed in the same TSK HW-55(S) column, PNGase F elute as the 10–12 h preparation.

The fact that PNGase F is less contaminated at the end of the log phase can be explained either by the lower amounts of Endo F present at this time (PNGase/Endo = 5) or the absence of contaminants due to cell lysis which is more important during stationary phase. Electrophoretic profiles (data not shown) confirmed this fact and showed that PNGase F was less contaminated, especially by the 43 kDa protein earlier described [5].

Recently, a new purification procedure for PNGase F and Endo F was reported using FPLC-controlled, hydrophobic-interaction chromatography on TSK-butyl and TSK-phenyl resins [17]. However, the earlier procedure, which is based on one step liquid chromatography [5, 6], can be used with the same good results when taking into account our observation that PNGase is less contaminated by other proteins at the end of the log phase.

The assay described in this paper is simple, reproducible and very sensitive. Using this assay, we demonstrated differential expression of PNGase F and Endo F during cultivation of *Flavobacterium meningosepticum.* Application of this new procedure will make easier the characterization and the quantification of endoglycosidases produced by other cell types.

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