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Note

Analysis of *N*-acetyl-chitooligosaccharides by the Iatroscan TLC/FID system

Margrethe Esaiassen *, Kersti Øverbø, Ragnar L. Olsen

Norwegian Institute of Fisheries and Aquaculture, P.O. Box 2511, N-9002 Tromsø, Norway

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At the present time two types of chitinolytic enzymes, chitinase (EC 3.2.1.14) and β-*N*-acetylhexosaminidase (EC 3.2.1.52), are recognised [1]. Chitinase randomly cleaves chitin polymers while the latter hydrolyses *N*-acetyl-D-glucosamine residues from nonreducing ends of chitobiose or higher *N*-acetyl-chitooligosaccharides. In addition, the existence of chitobiosidase, an exochitinase activity releasing dimeric units from chitin, has been reported [2]. Several methods can be used to a analyse the mode of action of chitinolytic enzymes. Chitooligosaccharides with a *p*-nitrophenyl group attached to the reducing end are often used as substrates. However, it has been reported that such substrates and the corresponding chitooligosaccharides give different enzymatic cleavage patterns [3]. Enzymatic hydrolysis of chitooligosaccharides can also be studied by HPLC and TLC techniques. In the TLC method the reaction products are often determined by visual inspection [4] or measured after recovering the separated sugars from the TLC plate [5]. In the HPLC method the oligosaccharides are usually separated on a NH_2-modified column and detected by UV absorbtion at 210–220 nm [6] or by a refractive index (RI) detector [7]. The method is relatively time consuming, only one sample is analysed at a time and the sample should be solubilised in the actual mobile phase. Carbohydrates do not posses specific UV-absorbing chromatophores. However, most carbohydrates do absorb in the near UV-range, 180–220 nm, but at this wavelength there is usually interference from other sample components [8]. The RI detector is the most commonly used in carbohydrate analysis, but the RI detector has a relatively low sensitivity, the minimum limit of detection of sample with the modern RI detector is

* Corresponding author.

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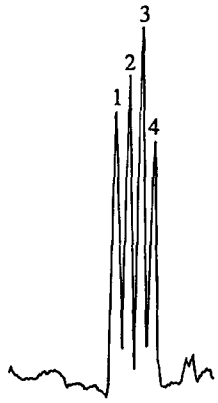


Fig. 1. TLC/FID chromatogram of a standard mixture of chitooligosaccharides. The sample (0.3 μL) contains 0.75 μg of each oligosaccharide. Peak 1, *N*-acetylglucosamine; peak 2, *N,N'*-diacetylchitobiose; peak 3, *N,N',N''*-triacetylchitotriose; and peak 4, *N,N',N'',N'''*-tetraacetylchitotetraose.

slightly lower than 10^{-6} g [9]. The Iatroscan TLC/FID which almost exclusively has been applied to the analysis of lipids [10,11], combines the efficiency of plate TLC for separations on a silica-gel coated Chromarod with the sensivity of the flame ionisation detection (FID). The Iatroscan TLC/FID system is equipped with sets of ten rods which give the possibility of analysing multiple samples, and the detection limit for the FID may be as low as 10^{-9} g [12].

In this report we describe the use of the Iatroscan TLC/FID system to analyse chitooligosaccharide standard solutions and heat-inactivated samples from the enzymatic hydrolysis of such oligosaccharides. Fig. 1 shows the chromatogram of the separated saccharides from a sample containing 0.75 μg of each of the standards; *N*-acetylglucosamine, *N,N'*-diacetylchitobiose, *N,N',N''*-triacetylchitotriose and *N,N',N'',N'''*-tetraacetylchitotetraose. With the exception of the tetrasaccharide, the mass response increased with the number of saccharide units present in the component. This is explained by the fact that the FID response is proportional to the number of oxidizable carbon atoms present [12]. The low response of the tetrasaccharide is caused by the low purity which according to the manufacturer, is only 75%. Using a computer program (Fig. P for Windows, Biosoft, Cambridge, UK), the results from the calibration with the tetrasaccharide gave a correlation coefficient of 0.999 for an exponential sigmoid model (Fig. 2). For a linear regression the correlation coefficient is 0.993. Similar correlation coefficients are found when calibrating with the other saccharides. For different lipid classes other authors have reported that there is a non-linear area response as a function of the amount of lipid present in the sample [11,13]. These results illustrate a potential drawback in the use of TLC/FID as a quantitative technique. However, within the narrow range of concentrations (0.75–6.0 μg) used in our work, the correlation coefficient for the linear regression model, is also good.

The TLC/FID analyses of the reaction products formed during the hydrolysis of *N,N',N'',N'''*-tetraacetylchitotetraose by chitinase from *Streptomyces griseus*, are shown in Fig. 3. The results show that the dimeric unit, chitobiose, is the main product of the

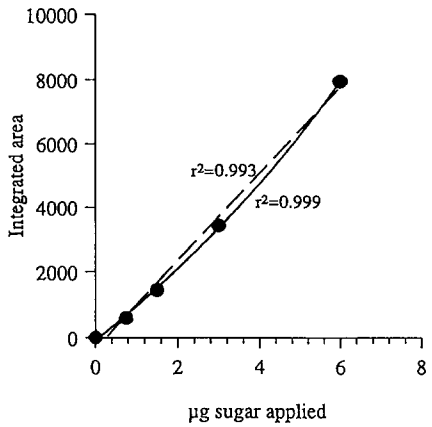


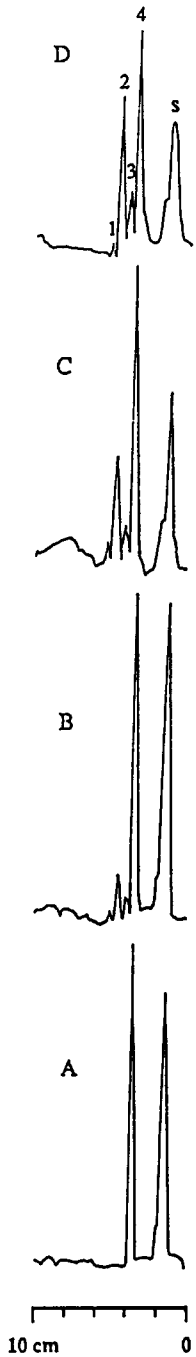
Fig. 2. Mass area response against the amount of *N,N′,N″,N‴*-tetraacetylchitotetraose applied to the rods. Solid line, an exponential sigmoid model; Broken line, a linear regression model.

hydrolysis. Since just a minor amount of *N*-acetylglucosamine is formed during the course of the reaction, the enzyme is not degrading the chitotetraose to chitobiose by first producing chitotriose. It is concluded that the enzyme degrades chitotetraose directly to chitobiose and can hence be designated a chitobiosidase. It is shown that TLC/FID is a convenient method for the separation and detection of chitooligosaccharides and may be useful in determining the product pattern of chitinolytic enzyme reactions.

1. Experimental

N-Acetylglucosamine, *N,N′*-diacetylchitobiose, *N,N′,N″*-triacetylchitotriose, *N,N′,N″,N‴*-tetraacetylchitotetraose, and chitinase from *Streptomyces griseus* (Sigma C 1525) were obtained from Sigma Chemical Co. All other reagents were of analytical grade. Thin layer chromatography with flame ionisation detection (TLC-FID) was performed on a Iatroscan TH-10 Mark IV equipped with Cromarod SIII, produced by Iatron Laboratories (Tokyo, Japan). Standards of *N*-acetylglucosamine, *N,N′*-diacetylchitobiose, *N,N′,N″*-triacetylchitotriose and *N,N′,N″,N‴*-tetraacetylchitotetraose were prepared in destilled water. Enzymatic degradation of chitooligosaccharides was performed using chitinase from *Streptomyces griseus* dissolved in water. The reaction mixture, consisting of 50 µL enzyme (0.1 mg/mL), 50 µL 25 mM sodium acetate buffer pH 5.5 and 100 µL of chitooligosaccharide (10 mg/mL), was incubated at 37°C.

Fig. 3. TLC/FID analyses of the reaction products after time-dependent incubation of *N,N′,N″,N‴*-tetraacetylchitotetraose with chitinase from *Streptomyces griseus*. The reaction mixture was analysed after A, 0 min; B, 10 min; C, 20 min; and D, 60 min. Peak 1, *N*-acetylglucosamine; peak 2, *N,N′*-diacetylchitobiose; peak 3, *N,N′,N″*-triacetylchitotriose; peak 4, *N,N′,N″,N‴*-tetraacetylchitotetraose; and peak S, sample application point.


D
4
2
s
3
1
C
B
A
10 cm
0

Aliquots were removed at various times and the enzyme was inactivated by heating on a boiling waterbath for 10 min followed by centrifugation.

Prior to sample application, the rods were cleaned and reactivated by a blank scan in the flame ionisation detection unit. Samples of 0.3 μL were applied on duplicate rods using a Hamilton syringe. The rods, ten in each experiment, were developed for 2.5 h in a solvent containing 2-propanol : ethanol : water : 25% ammonia (50:25:25:1) in an enclosed glass chamber. The chamber, lined with solvent-soaked filterpaper, had been equilibrated for 2 h before developing the rods. Subsequently the rods were dried at 105°C for 1.5 h before the flame ionisation detection scan. The detector was operated with an air flow of 2000 mL/min and a hydrogen flow of 160 mL/min. The scan speed was set at 3.3 mm/s and integration was carried out with a Spectra-Physics SP 4270 integrator.

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References

[1] E.C. Webb, Enzyme Nomenclature, IUBMB, Academic Press, San Diego, 1992.
[2] A. Tronsmo and G.E. Harman, *Anal. Biochem.*, 208 (1993) 74–79.
[3] D. Koga, K. Mizuki, A. Ide, M. Kono, T. Matsui, and C. Shimizu, *Agric. Biol. Chem.*, 54 (1990) 2505–2512.
[4] H. Blaak, J. Schnellmann, S. Walter, B. Henrissat, and H. Schrempf, *Eur. J. Biochem.*, 214 (1993) 659–669.
[5] K.M. Kragh, S. Jacobsen, and J.D. Mikkelsen, *Plant Science*, 71 (1990) 55–68.
[6] J. De La Cruz, A. Hidalgo-Gallego, J.M. Lora, T. Benitez, J.A. Pintor-Toro, and A. Llobell, *Eur. J. Biochem.*, 206 (1992) 859–867.
[7] V. Govindsamy and R. Balasubramanian, *Bot. Helv.*, 104 (1994) 79–86.
[8] L.M.L. Nollet (Ed.) *Food Analysis by HPLC*, Marcel Dekker, New York, 1994, pp 259–274.
[9] T. Shibamoto (Ed.) *Lipid Chromatographic Analysis*, Marcel Dekker, New York, 1994, pp 251–273.
[10] R.G. Ackman, C.A. McLeod, and A.K. Banerjee, *J. Planar Chromatogr.*, 3 (1990) 450–490.
[11] N.C. Shantha, *J. Chromatogr.*, 624 (1992) 21–35.
[12] H.H. Willard, L.L. Merritt, J.A. Dean, and F.A. Settle, *Instrumental Methods of Analysis*, 6th ed., Wadsworth Publishing Company, Belmont, California, 1981.
[13] J-L. Sebedio and P. Juaneda, *J. Planar Chromatogr.*, 4 (1991) 35–41.