Carbohydrate Research, 233 (1992) 273–278
Elsevier Science Publishers B.V., Amsterdam

Note

Properties of two β-glucosidases purified from the termite *Macrotermes muelleri* and from its symbiotic fungus *Termitomyces sp.*

Corrine Rouland [a], Mustapha Matoub [a], Philippe Mora [a] and Fahretin Petek [b]

[a] *Laboratoire de Biologie des Populations, Université Paris Val de Marne, 94010 Créteil (France)*
[b] *Laboratoire de Biologie Moléculaire de l'Interferon, ARC, B.P. 8, 94802 Villejuif (France)*

(Received May 22nd, 1991; accepted in revised form February 7th, 1992)

It is now well established[1] that degradation of native cellulose is dependent on the synergistic action of three types of enzymes, namely, endo-(1 → 4)-β-D-glucanases, cellobiohydrolases, and β-D-glucosidases.

In several insect species, it has been shown that the degradation of plant material in the digestive tract is accomplished with the assistance of enzymes synthesized by symbiotic microorganisms and ingested by the insect[2–5]. In previous papers, we demonstrated that in the digestive tract of *M. muelleri* (Termitidae, Macrotermitinae) the enzymes involved in cellulose degradation have two different sources. Of central importance are an endocellulase, produced by the symbiotic fungus *Termitomyces sp.* (Basidiomycetes, Agaricaceae) and ingested by the termites, and an exocellulase synthesized by the termites themselves[6–7]. The fungal enzymes, which act synergistically in the degradation of native cellulose[8], are efficient in two different organisms because of their great stability at various pHs and temperatures[6–7].

Two β-glucosidases were also purified previously, the first from the termite gut (β-glucosidase A) and the other from *Termitomyces sp.* (β-glucosidase B)[9], but their properties remained to be investigated. In this report, we describe some physicochemical properties of these two enzymes, which deserve emphasis because of their role in the digestion of cellulose by the termite *M. muelleri*.

MATERIALS AND METHODS

Biochemical products.—Avicellulose, carboxymethylcellulose, cellotriose, cellopentaose, cellobiose, and *p*-nitrophenyl β-D-glucopyranoside (PNPG) were ob-

Correspondence to: Dr. C. Rouland, Laboratoire de Biologie des Populations, Université Paris Val de Marne, 94010 Créteil Cedex, France.

tained from Sigma Chemical Co. (St Louis, MO, USA). Bovine serum albumin originated from Koch-Light Laboratories (Colnbrook, Bucks, UK). Phosphorylase *a* and pepsin were from Worthington Biochemical Corp. (Freehold, NJ, USA) and trypsin was from Armour Laboratories (London, UK).

Enzyme preparations.—β-Glucosidases A and B were purified by ion-exchange chromatography as detailed in a previous paper[9]. Each purified enzyme gave a single protein band on gel electrophoresis under nondenaturing conditions. The specific activities of the two enzymes were respectively 440 U per mg protein for β-glucosidase A and 90 U per mg protein for β-glucosidase B with PNPG as substrate. On cellobiose, the values were 398 U per mg protein for β-glucosidase A and 207 U per mg protein for β-glucosidase B.

Enzyme assays.—Cellobiase and β-glucosidase activities were assayed by incubating 50 μL of purified enzyme solution with 50 μL of cellobiose or PNPG (50 mM) and 25 μL of McIlvaine buffer[10]. The release of glucose during the hydrolysis of cellobiose was determined by a microdosage technique using Glucosoxidase[11]. Proteins were determined by the Coomassie Brilliant Blue G250 technique[12] using bovine serum albumin as a standard. Specific activity was expressed as μmol of glucose liberated per min per mg of protein at 37° (U per mg protein).

Gel electrophoresis.—Molecular weight determinations were performed on polyacrylamide disc gels[13] using the Hedrick and Smith method[14] and by SDS-PAGE following the procedure described by Weber and Osborn[15]. Reference proteins used were phosphorylase *a* monomer (94 kDa), bovine serum albumin monomer (68 kDa), pepsin (35 kDa), and trypsin (23.3 kDa).

RESULTS

Molecular weights.—The electrophoretically pure enzymes each gave a single protein band corresponding to $m = 126 \pm 11$ kDa for β-glucosidase A and $m = 115 \pm 10$ kDA for β-glucosidase B. The apparent molecular masses of β-glucosidase A and B, determined by SDS-PAGE, were respectively 62 ± 2 kDa and 120 ± 4 kDa. This suggests a dimeric structure for β-glucosidase A and a monomeric structure for β-glucosidase B.

Glycoprotein nature.—The two β-glucosidases were tested for carbohydrate by periodic acid–Schiff staining after polyacrylamide disc gel electrophoresis. Only β-glucosidase A presented a positive response to periodate–fuschin, suggesting a glycoprotein nature for this enzyme.

Enzyme specificity.—Both enzymes were active on cellobiose and PNPG, but they showed no activity on microcrystalline cellulose, avicellulose, carboxymethylcellulose (CMC), gentiobiose (β1-6), or laminarabiose (β1-3). β-Glucosidase A also hydrolyzed cellotriose and cellopentaose with specific activities of respectively 50 U per mg protein and 29 U per mg protein.

Effect of pH on rates of hydrolysis of cellobiose and PNPG.—Rate curves of the hydrolysis of PNPG by β-glucosidases A and B showed linearity up to 15 min,

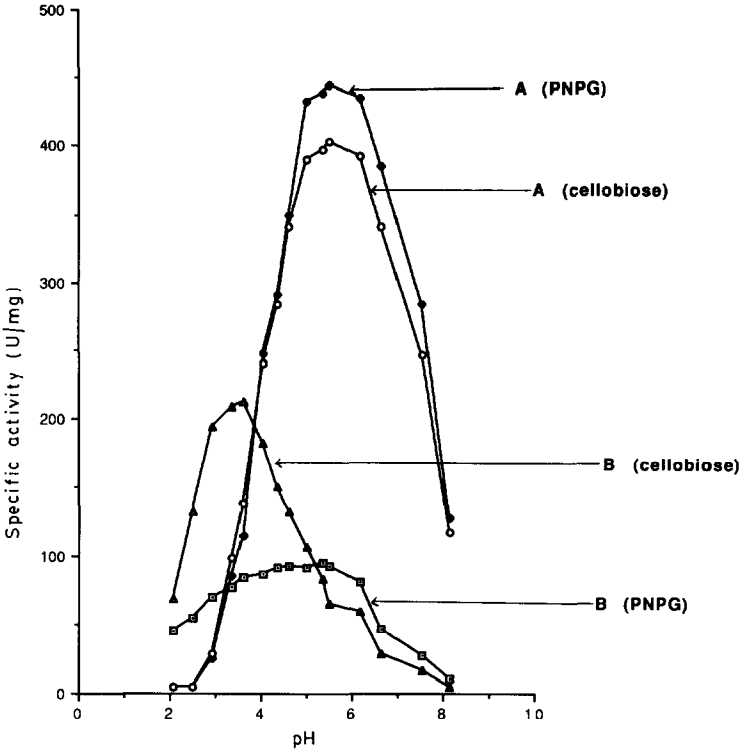

Fig. 1. Effect of pH on rates of cellobiose and PNPG hydrolysis by β-glucosidases A and B. Each point is the mean value of three experiments.

whereas the hydrolysis of cellobiose was linear up to 10 min for β-glucosidase A and up to 20 min for β-glucosidase B. Therefore, an incubation time of 10 min was used for all subsequent experiments.

The effect of pH on the activity of the β-glucosidases was studied at 37° by using cellobiose or PNPG as substrate in McIlvaine buffer over the pH range 2.8–8. The pH-activity profiles obtained (Fig. 1) were very different for the two enzymes. β-Glucosidase A showed a maximum of activity at pH 5.3 for both substrates. By contrast, β-glucosidase B showed a maximum between 4.7 and 5.3 on the heteroside (PNPG), whereas the optimum pH was between 2.95 and 3.3 with cellobiose as substrate.

Thermal denaturation.—Both enzymes could be stored at 4° for several months without any apparent loss of activity. However, β-glucosidases A and B lost respectively 10 and 30% of their activities on freeze-drying.

Temperature stability was measured by incubating aliquots (0.05 mL) of enzyme in McIlvaine buffer (pH 5.3) at 30–55° for 20 min. Residual activity on PNPG and on cellobiose was then assayed at 37° (Fig. 2). β-Glucosidase A was less heat sensitive than β-glucosidase B; on preincubation at 50° for 20 min it lost 30% of its activity on cellobiose and 60% of its activity on PNPG. Under the same conditions,

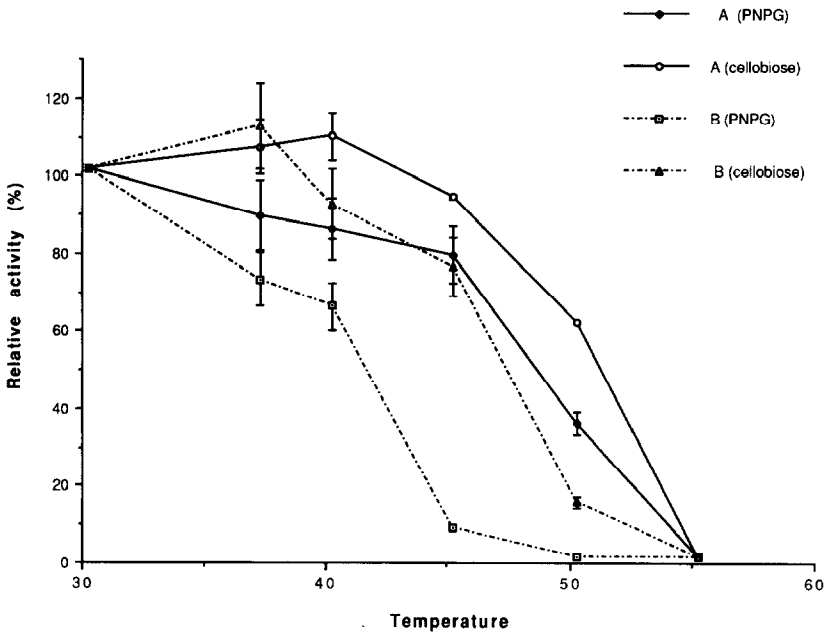

Fig. 2. Temperature stability of β-glucosidases A and B, tested with cellobiose and PNPG as substrates. Each point was calculated from the average value of five tests.

β-glucosidase B lost 100% of its activity on the heteroside and 80% on cellobiose. Moreover, both β-glucosidases were lightly activated after a preincubation at 37°, but only when assayed on cellobiose.

Effect of substrate concentration.—The effect on the reaction rate of varying substrate concentration was studied with PNPG and cellobiose. The apparent Michaelis constant (K_m) for each enzyme was calculated from Lineweaver–Burk plots[16]. On cellobiose, values of K_m for β-glucosidases A and B were respectively $1 \pm 0.05 \times 10^{-3}$ M and $2.8 \pm 0.2 \times 10^{-3}$ M. With PNPG as substrate β-glucosidase A had a K_m of $1.3 \pm 0.2 \times 10^{-3}$ M, which was similar to that on cellobiose. The K_m of β-glucosidase B on PNPG could not be established because of the very low affinity of the enzyme for this substrate. For β-glucosidase A the K_m could be also determined on cellotriose and cellopentaose; the values obtained were respectively $1.1 \pm 0.09 \times 10^{-3}$ M and $7.7 \pm 0.4 \times 10^{-3}$ M.

Effect of various cations.—Enzyme activity was measured under the standard assay conditions with cation concentrations up to 0.02 M. No significant effect on the two β-glucosidases was observed with Mn^{2+}, Co^{2+}, Mg^{2+}, or Ca^{2+}. Under the same conditions, β-glucosidase A was inactivated by Ag^{+}, Fe^{2+}, Cu^{2+}, and Zn^{2+}. It also lost 50% of its cellobiase activity with EDTA at 10^{-3} M. After dialysis to remove EDTA, each of the cations just mentioned, and also Pb^{2+} and Li^{+}, was tested for its ability to restore the activity, but none had any effect. Except for Ag^{+} and Cd^{2+}, cations had no apparent effect on the activity of the β-glucosidase B,

which was also definitely inhibited by EDTA. In this case, the addition of Fe^{2+} restored 75% of the initial enzymic activity.

DISCUSSION

β-Glucosidase A purified from the gut of *Macrotermes muelleri* and β-glucosidase B isolated from its symbiotic fungus *Termitomyces sp.* are very different in structure. β-Glucosidase A is a homodimer of *m* 62 kDa, whereas β-glucosidase B is a monomer of *m* 115 kDa. Only β-glucosidase A appeared to be a glycoprotein. The optimal pH values (5.2 and 5) are comparable to those of other β-glucosidases from insects[17,18] or fungi[19,20]. Both enzymes are heat sensitive, with their cellobiase activities better conserved than their β-glucosidase activities during preincubation at high temperature. This result in the case of β-glucosidase B could be correlated with the greater affinity of the enzyme for cellobiose than for PNPG.

β-Glucosidase A is more sensitive to metal ions than β-glucosidase B. The observation with β-glucosidase B that activity lost with EDTA is restored by Fe^{2+} suggests that ferrous ion may be a component of the active site of this enzyme.

The strict specificity of the two β-glucosidases for cello-oligosaccharides and PNPG puts these enzymes in contrast to those from *Trichoderma viride*[21], *Aspergillus roseus*[22], and *Ergates faber*[17], which are also active on microcrystalline cellulose and CMC, or that of *Sclerotium rolfsii*, which degrades glucan substrates having β-(1 → 6) and β-(1 → 3) linkages[23]. As judged by the K_m value, β-glucosidase B has a lower affinity for cellobiose than other fungal β-glucosidases[24]. The fact that this enzyme showed no activity on all the other tested substrates (CMC, cellotriose, cellopentaose, and cellulose) indicates that it is a strict cellobiase (a β-D-glucoside glucohydrolase, EC 3.2.1.21.). The series of K_m values obtained for β-glucosidase A with PNPG, cellobiose, cellotriose, and cellopentaose suggest that the affinity of the enzyme decreases as the substrate molecular weight increases, in agreement with the results reported for the β-glucosidases of *A. roseus*[22] or *T. koningii*[25].

Although the symbiotic *Termitomyces* is strongly cellulolytic, it possesses only an endocellulase and a lightly active β-glucosidase. Thus, it may have the same cellulolytic metabolism as the brown-rot fungi. These fungi, which have very little β-glucosidase activity, use an oxidative system (H_2O_2–Fe^{2+}) for the degradation of cellobiose[26].

β-Glucosidase A, released by termite salivary glands[27], is much more active than the fungal enzyme. The clear activity of β-glucosidase A on cellotriose and cellopentaose explains the great increase in cellulose hydrolysis when the three enzymes (endocellulase produced by the fungus, exocellulase and β-glucosidase synthesized by the termite) act synergistically in the termite digestive tract[8]. It appears that the terminal stages of the cellulolytic pathway in *Macrotermes muelleri* and in its symbiotic fungus are clearly different.

REFERENCES

1 T.M. Wood and S.I. McCrae, in R.D. Brown, Jr. and L. Jurasek (Eds.), *Hydrolysis of Cellulose: Mechanisms of Enzymatic and Acid Catalysis*, Adv. Chem. Ser. 181, American Chemical Society, Washington, DC, 1979, pp 181–209.
2 T.G. Wood and R.J. Thomas, in N. Wilding, N.M. Collins, P.M. Hammond, and J.F. Weber (Eds.), *Insect – Fungus Interactions*, Academic Press, London, 1989, pp. 24–82.
3 J.J. Kukor and M.M. Martin, *Science*, 220 (1983) 1161–1163.
4 J.J. Kukor and M.M. Martin, *Oecologia*, 69 (1986) 360–366.
5 J.J. Kukor, D.P. Cowan, and M.M. Martin, *Physiol. Zool.*, 61 (1988) 364–371.
6 C. Rouland, A. Civas, J. Renoux, and F. Petek, *Comp. Biochem. Physiol., B: Comp. Biochem.*, 91 (1988) 449–458.
7 C. Rouland, J. Renoux, and F. Petek, *Insect Biochem.*, 18 (1988) 709–715.
8 C. Rouland, A. Civas, J. Renoux, and F. Petek, *Comp. Biochem. Physiol., B: Comp. Biochem.*, 91 (1988) 459–465.
9 C. Rouland, P. Mora, M. Matoub, J. Renoux, and F. Petek, *Act. Coll. UIEIS*, 4 (1986) 109–118.
10 T.C. McIlvaine, *J. Biol. Chem.*, 49 (1921) 183–186.
11 W. Werner, H.G. Rey, and H. Wiellinger, *Anal. Chem.*, 252 (1970) 224–228.
12 J.J. Sedmak and S.E. Grossberg, *Anal. Biochem.*, 79 (1977) 544–552.
13 J.V. Maizel, *Ann. N.Y. Acad. Sci.*, 121 (1964) 381–390.
14 J.L. Hedrick and A. Smith, *Arch. Biochem. Biophys.*, 126 (1968) 155–165.
15 K. Weber and M. Osborn, *J. Biol. Chem.*, 244 (1969) 4406–4412.
16 H. Lineweaver and K. Burk, *J. Am. Chem. Soc.*, 56 (1934) 658–666.
17 J.M. Chipoulet and C. Chararas, *Comp. Biochem. Physiol., B: Comp. Biochem.*, 80 (1985) 241–246.
18 C. Chararas and J.M. Chipoulet, *Comp. Biochem. Physiol., B: Comp. Biochem.*, 72 (1982) 559–564.
19 M. Desrochers, L. Jurasek, and M.G. Paice, *Appl. Environ. Microbiol.*, 41 (1981) 222–228.
20 A. Margaritis and R. Merchant, *Biotechnol. Bioeng.*, 13 (1983) 299–314.
21 L.H. Li, R.M. Flora, and K.W. King, *Arch. Biochem. Biophys.*, 111 (1965) 439–447.
22 G. Vodjani, *Thesis*, U. Paris VI, 1981, pp 2–145.
23 J.C. Sadana, J.G. Shewale, and R.V. Patil, *Carbohydr. Res.*, 118 (1983) 205–214.
24 D.A.J. Wase, S. Raymahasay, and C.W. Wang, *Enzyme Microb. Technol.*, 7 (1985) 225–229.
25 T.M. Wood and S.I. McCrae, *J. Gen. Microbiol.*, 128 (1982) 2973–2982.
26 A. Mouranche and C. Costes, *Hydrolases et Depolymerases*, Gauthier-Villars, (1985).
27 C. Rouland, J.J. Lenoir-Rousseaux, P. Mora, and J. Renoux, *Sociobiology*, 15 (1988) 237–246.