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Effect of Some Inhibitors on the Activity of Cellobiose Phosphorylase and Cellotriose Phosphorylase in *Ruminococcus flavefaciens* 17

[1]Sobhy M.A. Sallam, [1]M.E.A. Nasser; [2]K.Ushida, [2]K. Miyazaki and [1]A.M.El-Waziry

[1]Animal Production Department, Faculty of Agriculture,
Alexandria University, Alexandria, El-Shatby, Egypt
[2] Laboratory of Animal Science, Kyoto Prefectural University, Kyoto, Japan.

Abstract: The present study was examined the effects of some inhibitors on the activities of cellobiose phosphorylase (CBPase) and cellotriose phosphorylase (CTPase) in *Ruminococcus flavefaciens* 17. Three levels, 2, 4 and 6 mM, of heavy metals, Mn^{2+}, Co^{2+}, Zn^{2+}, Ni^{2+} and Cu^{2+}, were tested. Also, Various levels of the ethylinediaminetetracetic acid (EDTA), 1,10-o-phenanthroline, L-cycteine, *p*-chloromercuribenzoate (PCMB), *N*-Ethylmaleimide, Iodoacetate and Phenylmethylsulphonyl fluoride (PMSF) were investigated. The activity of CBPase and CTPase in *R.flavefaciens* 17 was completely inhibited by heavy metals at 2, 4, 6 mM. While, PCMB completely inhibited the activity of CBPase but 50% inhibition occurred in CTPase. The activity of CBPase and CTPase was decreased by 12-16% with EDTA or PMSF. The L-cycteine or 1,10-o-phenanthroline caused slight inhibition for CBPase and CTPase activities. *N*-Ethylmaleimide (0.5 mM) had little effect on both phosphoryases but 1.5 mM of N-Ethylmaleimide resulted in 27% or 38% inhibition for CBPase and CTPase activity. When used 5.0 mM of iodoacetate, the CBPase activity decreased 7% and CTPase activity decreased 16%, while 7.5 mM of Iodoacetate caused 18 and 27% of decline for CBPase and CTPase activities, respectively. Addition of thiol-blocking agents at 20µM of PCMB caused 100% inhibition for CBPase activity.

Key words: Rumen, Cellulolytic bacteria, Phosphorylases activity

INTRODUCTION

Ruminants can change the plant cell walls to protein food (meat, milk, cheese etc.) by the help of microorganisms, which habits in their rumen. Among them, contribution of the cellulolytic bacteria to this function is important. Cellulose degradation is realized by mixture of cellulolytic enzymes, such as endo-b-1,4-glucanase, cellobiohydrolase and â–glucosidase. Phosphorylase enzymes catalyze the reversible cleavage of polysaccharides, oligosaccharides, or disaccharides into á-D-glucose-1-phosphate and hence play a central role in carbohydrate metabolism. The information on phosphorolysis will help elucidate its potential role in cellulolysis and is also necessary for a comprehensive understanding of carbohydrate utilization by rumen bacteria. Cellulolytic bacteria usually degrade cellulose by the synergistic action of endo- and exo-glucanase[13,14], but this process is potentially inhibited by soluble end products[8].

Ruminococcus flavefaciens is one of the most predominant cellulolytic bacteria isolated from the rumen. The physiology, enzymology and genetics of cellulose degradation by this bacterium have been studied, but many question remained unanswered. The cellulolytic enzyme system from *R. flavefaciens* has been examined in some depth and has been shown to be complex, involving numerous enzymes. The main end products of cellulolysis by *R. flavefaciens* are cellotriose and cellobiose, with only small amounts of glucose being produced[15,17].

In this bacterium the existence of Phosphotransferase system (PTS) was not reported so far and the activity of cellobiose phosphorylase was detected[2] The finding of phosphorylases in this organism is very interesting and this will compensate the lack of PTS system because there are similarity between PTS and phosphorolysis. The phosphoroylysis is genetically advantageous and constitute the primary route of cellooligosacchrides utilization, particularly in anaerobic environment and initiate the Embden-Meyerhoff-Parnas pathway by cellobiose phosphorylase. Cellobiose phosphorylases are interesting enzymes, for the energy of the â-1,4 glycosidic bond is conserved during phosphorolysis in the form of glucose 1-phosphate, therefore, the objective of this study was to investigate the effect of some inhibitors on cellobiose phosphorylase or cellotriose phosphorylase activity in *R. flavefaciens* 17.

Corresponding Author: Sobhy M.A.Sallam, Department of Animal Production, Faculty of Agriculture, Alexandria University, Alexandria,El-Shatby, Egypt.
E-mail : s_sallam@yahoo.com

MATERIALS AND METHODS

The present study was carried out at the Kyoto Prefectural University; Laboratory of Animal Science; Kyoto; JAPAN.

Bacterials strain and culture media: *Ruminicoccus flavefaceins* 17 kindly provided by H.J.Flint, Rowett Research Institute, Greenburn, Bucksburn, Aberdeen AB2 9SB, UK. It was grown anaerobically at 37°C in M_2 medium[3,5].

Cell fractionation: The cells were harvested by centrifugation (15000 rpm for 10 min at 4°C). The pellet was washed two times with either 50 mM Tris-HCl buffer plus 2 mM dithiothreitol (pH 6.8, 4°C). The pellets suspended in the buffer. The suspension ultarsonicated (20 times, 30 sec.) and centrifuged for 10 min, 15000 rpm at 4°C for enzyme assays[7,11].

Inhibitors: Various levels of inhibitors were used to investigate their effects on the cellobiose and cellotriose phosphorylase activity in *Ruminicoccus flavefaceins* 17. The inhibitors used were 5, 10 or 15 mM of eithylinediaminetetracetic acid (EDTA), 0.5, 1.0 or 1.5 mM of 1,10-o- phenanthroline, 1.0, 2.0 or 3.0 mM L-cysteine, 10,20 or 30 μM of *p*-chloromercuribezoate (PCMB), 0.5,1.0 or 1.5 mM of *N*-Ethylmaleimide, 2.5, 5.0 or 7.5 mM of Iodoacetate and 0.75, 1.5 or 2.25 mM of Phenylmethylsulphonyl fluoride (PMSF). Also 2.0, 4.0 or 6.0 mM of Cobalt, Cupper, Manganese, Nickel or Zinc were used.

Enzyme assays: Cellobiose and cellotriose phosphorylase activities assays were also performed by measuring Pi formation[1] as follows:

Cellobiose phosphorylase assay

Reagent: 0.2 M, D-Glucose 1-phosphate, sodium salt, (pH 6.8), L-Cysteine, 80 mM, pH 6.8, EDTA, 4.0 mM, pH 6.8 and Acetic acid, 0.1 M

Procedure: Before the assay, the enzyme is incubated in 20mM cysteine and 1.0 mM EDTA, pH 6.8, for 5 minutes at 37°C. The reaction mixture in a total volume of 1.0 ml contains 0.2 ml of glucose 1-phosphate, 0.2 ml D-xylose, 0.2 ml cysteine, 0.2 ml EDTA and 0.2 ml of pre-incubated enzyme. In one tube the reaction is terminated by adding 7 ml of acetic acid before adding the pre-Incubation enzyme (zero time). In the other tubes the reaction terminated after 15 min by the addition of acetic acid. The amount of Pi liberated is determined by the Fisk-SubbaRow method[4].

Definition of Unit and Specific Activity: unit of cellobiose phosphorylase is defined as the amount which catalyzes the liberation of 1 μmole of Pi per 15 minutes under the conditions described. Specific activity is expressed a units per milligram of protein. Protein determined by procedure of Lowery[12].

Cellotriose Phosphorylase assay

Reagents: 0.4 M, D-Glucose 1-phosphate, sodium salt, (pH 6.8), Cellobiose, 0.1M, L-Cysteine, 80 mM, pH 6.8, EDTA, 4 mM, pH 6.8 and Acetic acid, 0.1 M 0.4 M, D-Glucose 1-phosphate, sodium salt, (pH 6.8Cellobiose, 0.1M, Dithiothreitol, 0.16M, EDTA, 0.16mM, pH 6.8, Tris, 0.16M and Acetic acid, 0.1 M

Procedure: Before the assay, the enzyme is incubated with 40 mM DTT, 20 mM Tris and 20 mM EDTA, pH 6.8, for 30 minutes at 37°C. The reaction is started by addition of 0.8 ml of a mixture containing 0.2 ml of glucose 1-phosphate, 0.2 ml of cellobiose, 0.2 ml of DTT, 0.0375 ml of Tris, .0375 ml of Tris, 0.0375 ml of EDTA and 0.125 ml of water to 0.2 ml of the pre Incubated enzyme solution. The reaction is terminated immediately in one sample by addition of 0.2 ml of reaction mixture to 7 ml of acetic acid. After incubation at 37°C for 15 minutes, the reaction is terminated in a similar manner. The amount of Pi liberated is determined by the Fiske-SubbaRow method[4].

Definition of unit and specific activity: One unit of cellotriose phosphorylase is defined as the amount which catalyzes the liberation of 1 μmole of Pi per 15 minutes under the conditions described. Specific activity is expressed a units per milligram of protein. Protein determined by procedure of Lowery[12].

Statistical analysis: The statistical analysis was carried out according to Snedecor and Coshran[19].

RESULTS AND DISCUSSION

Influence of various level of potential inhibitors on CBPase and CTPase activities in *R.flavefaciens* 17 are shown in Table 1, Fig. 1 and 2. EDTA (0.5mM) was slightly affected on the activities of CBPase and CTPase, while 15 mM of EDTA caused inhibition about 13% and 16%, respectively for CBPase and CTPase production. No inhibition for CBPase was observed when L-cysteine was added and slight decrease in the activity of CTPase was observed. PCMB (10 μM) resulted in 80% inhibition for CBPase activity and 20% for CTPase activity. PCMB (20,30μM) inhibited completely the activity of CBPase.

N-Ethylmaleimide (0.5 mM) had little effect on both phosphoryases but 1.5 mM of *N*-Ethylmaleimide resulted in 27 or 38% inhibition for CBPase and CTPase activity. While *N*-Ethylmaleimide at 0.1, 1.0, or 10 mM inhibited the CBPase by 44, 53 and 91% respectively but dithioteritol (DTT) increased the activity of CBPase by

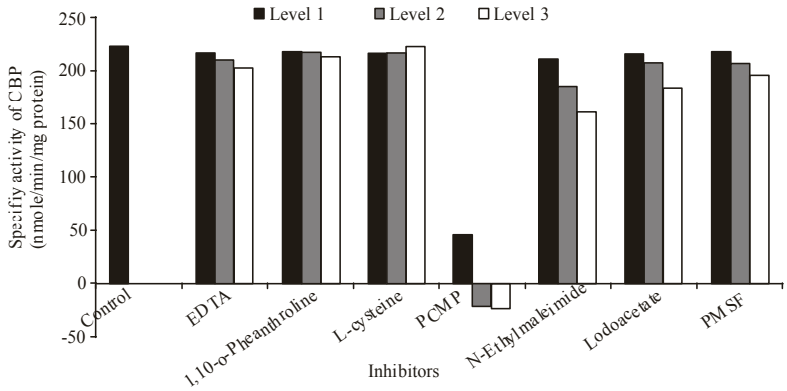

Fig. 1: Effect of various levels of some inhibitors on the cellobiose phosphorylase activity in *R. flavefaciens* 17

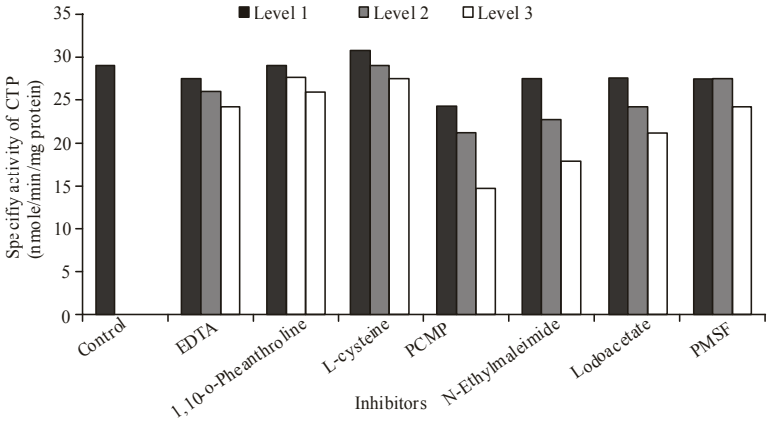

Fig. 2: Effect of various levels of some inhibitors on the cellotriose phosphorylase activity in *R. flavefaciens* 17

Table 1: Inhibitory effect for the different levels of inhibitors on the cellobiose phosphorylase CBPase and cellotriose phosphorylase CTPase activities in *R.flavefaciens* 17 grown on cellobiose.

Inhibitor	Concentration (mM or µM)[a]	Relative activity of CBPase (%)	Relative activity of CTPase (%)
None		100	100
EDTA	5	98	94.8
	10	94.3	89.4
	15	86.5	83.9
1.10-*o*-phenanthroline	0.5	97.9	96.7
	1	97.8	94.9
	1.5	95.7	89.3
L-cycteine	1	97.2	105.7
	2	97.9	100.2
	3	100	94.8
PCMB	10	20.9	83.9
	20	0	72.9
	30	0	51.1
N-Ethylmaleimide	0.5	94.3	94.8
	1	83.6	78.4
	1.5	72.9	62
Iodoacetate	2.5	97.1	94.8
	5	92.9	83.9
	7.5	82.9	72.9
PMSF	0.75	98.6	94.8
	1.5	92.9	94.8
	2.25	87.9	83.9

a; µM for PCMP, all others inhibitors in mM order.
CBPase, Cellobiose phosphorylase, CTPase, Cellotriose phosphorylase
EDTA, Ethylendiaminotetra acetic acid,
PCMB, *p*-chloromercuribezoate
PMSF, Phenylmethylsulphonyl fluoride

37, 66 and 85% at 1, 5 and 10 mM in *Clostridium thermocellum*[20]. In addition, 500 µM of *N*-Ethylmaleimideresulted in a 56% inactivation of CBPase and 100% inhibition at 20 µM PCMB in *Cellvibrio gilvus*[18]. These results suggested that there are exposed residues and thiol groups essential for CBPase activity. Iodoacetate at 2.5 mM slightly decreased the CBPase activity but CTPase lost 5% from its activity. When used 5.0 mM of iodoacetate, the CBPase activity decreased 7% and CTPase activity decreased 16%, while 7.5 mM of Iodoacetate caused 18 and 27% of decline for CBPase and CTPase activities, respectively. The CBPase activity was inhibited by nojirimycin but weakly inhibition occurred with α-Oxogluconate, glucono-δ-lactone, methyl-α-D-glucoside, 6-phosphogluconate or ρ-Nitrophenyl ß–glucopyranoside in Cellvibrio gilvus[18]. Nojirimycin is known as an inhibitor of ß-glucosidase which is produced by S*treptomyces roseochromogenes*[6].

The D-glucose substrate inhibited CBPase activity in *Cellvibrio gilvus, Clostridium thermocellum* and *Thermotoga maritima.* This inhibition were explained as competitive substrate in which one substrate namely D-glucose, competitively inhibited the other substrate, glucose-1-phosphate[9,10,16,20]. When cellobiose, glucose, lactose or maltose were used as a sole energy source in

another study, cellobiose phosphorylase activity was induced by cellobiose in *R.flavefaciens* 17 but the activity was low in glucose-grown cells indicating that the enzyme was regulated by the carbon source and induced by either lactose or cellobiose[22]. In addition, cellulose, cellodextrins, cellobiose or xylan were used as a sole energy source in *R.flavefaciens* 17 and showed that the activity of cellobiose phosphorylase in cellulose-grown cells of *R.flavefaciens* 17 was low. Highest activity was found in cellobiose-grown cells indicating that *R.flavefaciens* 17 had high affinity and preference for cellobiose, The highest bacterial growth rate was found in cellobiose-grown cells after 18 hr incubation and at the same time from 18-21 hr incubation, the highest activity for cellobiose phosphorylase was observed indicating that production of such enzyme encourages the bacterial growth rate. The high growth yield on cellobiose could be attributed to the action of cellobiose phosphorylase[21,22]

There was no effect observed for 0.75 mM of PMSF on CBPase but slightly decreased the CTPase activity by 0.75 or 1.5 mM of PMSF. While 1.5 or 2.25 mM PMSF were slightly affected on the CBPase activity. Three levels, 2, 4 and 6 mM, of heavy metals, Mn^{2+}, Co^{2+}, Zn^{2+}, Ni^{2+} and Cu^{2+}, were tested. All these heavy metals were completely inhibited the CBPase and CTPase activities in *R.flavefaciens* 17 at any dose levels. On the other hand, there is no marked effect for some metal ions at 1.0 mM concentration on the CBPase activity in *Clostridium thermocellum*[20]. The complete inhibition caused by heavy metals may be due to high level incorporated in this study. The phopshorylases produced by cellulolytic bacteria are urgent for prevention of the inhibition of cellulolysis in the rumen and chemical inhibitors should be consider as inhibiting agents for such important degrading enzymes of cellulose end products. These results suggest that there are exposed residues very important for CBPase activity. However, little work has been done on these enzymes and more studies are required on the activity of phosphorylase in cellulytic rumen bacteria.

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