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SCIENCE DIRECT
Enzyme and Microbial Technology 39 (2006) 281–288
ENZYME AND MICROBIAL TECHNOLOGY
www.elsevier.com/locate/emt

Effect of condensed tannins from tropical legumes on the activity of fibrolytic enzymes from the rumen fungus *Neocallimastyx hurleyensis*

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Received 30 May 2005; accepted 25 October 2005

Abstract

A study was conducted to assess the effect of condensed tannins on the activity of fibrolytic enzymes from the anaerobic rumen fungus, *Neocallimastix hurleyensis* and a recombinant ferulic acid esterase (FAE) from the aerobic fungus *Aspergillus niger*. Condensed tannins were extracted from the tropical legumes *Desmodium ovalifolium*, *Flemingia macrophylla*, *Leucaena leucocephala*, *Leucaena pallida*, *Calliandra calothyrsus* and *Clitoria fairchildiana* and incubated in fungal enzyme mixtures or with the recombinant FAE. In most cases, the greatest reductions in enzyme activities were observed with tannins purified from *D. ovalifolium* and *F. macrophylla* and the least with tannins from *L. leucocephala*. Thus, whereas 40 $\mu g\,ml^{-1}$ of condensed tannins from *C. calothyrsus* and *L. leucocephala* were needed to halve the activity of *N. hurleyensis* carboxymethylcellulase (CMCase), just 5.5 $\mu g\,ml^{-1}$ of the same tannins were required to inhibit 50% of xylanase activity. The β-D-glucosidase and β-D-xylosidase enzymes were less sensitive to tannin inhibition and concentrations greater than 100 $\mu g\,ml^{-1}$ were required to reduce their activity by 50%. In other assays, the inhibitory effect of condensed tannins when added to incubation mixtures containing particulate substrates (the primary cell walls of *F. arundinacea*) or when bound to these substrate was compared. Substrate-associated tannins were more effective in preventing fibrolytic activities than tannins added directly to incubations solutions. It was concluded that condensed tannins from tropical legumes can inhibit fibrolytic enzyme activities, although the extent of the effect was dependent on the tannin, the nature of its association with the substrate and the enzyme involved.
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Keywords: Condensed tannins; Tropical legumes; Fibrolytic enzymes; Ferulic acid esterase; Anaerobic fungi

1. Introduction

Ruminants meet their energy requirements through the degradation of dietary fibre, which principally occurs through the activity of fungal, protozoal and bacterial polysaccharidases [1]. Anaerobic fungi have been reported to play a significant role as the primary colonisers of plant biomass in the rumen [2,3]. They possess some of the most potent fibrolytic enzymes known [4], have been shown to degrade fibrous particles of dissimilar size at similar rates [3] and can penetrate and colonise recalcitrant cell walls that are resistant to bacterial attack [5].

Polyphenolic, anti-nutritional factors in plants can influence the activity of polysaccharidases in the rumen. Condensed tannins, polyphenols with the ability to bind and precipitate proteins, carbohydrates and other macromolecules [6], are abundant in leguminous forages and can limit ruminal fibre degradation in both temperate [7,8] and tropical [9,10] livestock. These effects are probably mediated via the reduction of ruminal microbial polysaccharidase activity or because of the tannin's precipitation activity, thereby decreasing the susceptibility of substrates to colonization and degradation by rumen micro-organisms [10,11].

Condensed tannins are heterogeneous macromolecules and molecular weight can affect their ability to precipitate proteins [12–15]. Given that condensed tannins occur in considerable abundance in tropical forage legumes, especially in those adapted to acid, low-fertility soils, this work aimed to study their effects on the activity of *N. hurleyensis* polysaccharidases

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0141-0229/$ – see front matter © 2005 Elsevier Inc. All rights reserved.
doi:10.1016/j.enzmictec.2005.10.011

(cellulases, xylanases and β-glycosidases) and a recombinant ferulic acid esterase (FAE from *A. niger* provided by Genencore International Ltd. and used as a 'mimic' of an anaerobic fungal esterase). The hypotheses that we wished to address were two-fold, namely (a) that tannins from different legumes and with different chemistries would inhibit the same enzymes to markedly differing extents and (b) that tannins from the same legume would inhibit different enzymes to markedly differing extents. In addition, we wanted to compare the extent of the inhibitory effect displayed by tannins when they were added to reaction solutions containing particulate substrates, or when they were bound to the cell walls of particulate substrates.

2. Materials and methods

2.1. Condensed tannin source

Condensed tannins were extracted from mature leaves of the tropical legumes *D. ovalifolium*, *F. macrophylla*, *L. leucocephala*, *L. pallida*, *C. calothyrsus* and *C. fairchildiana*, and purified according to the method reported [11]. One experiment also made use of condensed tannins extracted from the temperate legume, *L. corniculatus*, extracted as reported in [11].

2.2. Fungal culture techniques

N. hurleyensis was maintained in batch culture at 39 °C in gas-tight serum bottles in modified medium C [16]. The fungus was sub-cultured at 4–5 day intervals. Carboxymethylcellulase (CMCase) and β-D-glucosidase activities were enriched by culturing the fungus on milled wheat straw, while xylanase and β-D-xylosidase activities were enriched by culturing on oat spelt xylan (Sigma X0627) [17]. All batch cultures were prepared and grown without agitation using anaerobic procedures. Experimental cultures were sub-cultured on modified medium C containing the appropriate substrate for at least three transfers prior to using their culture supernatants as an enzyme source.

2.3. Enzyme source

Fungal culture supernatants from experimental cultures were harvested after 4–5 days of incubation. Culture supernatants were centrifuged for 5 min (11,000 g at room temperature, Microcentaur micro-centrifuge) and the clarified enzyme solutions kept at −70 °C until needed for enzyme assays, which were conducted in the presence and absence of condensed tannins. In addition to these enzyme solutions, the effect of condensed tannins on the activity of a recombinant FAE was investigated. The enzyme source for all enzyme reactions was a solution of the appropriate enzyme diluted with citrate phosphate buffer (1:1, v/v).

2.4. Enzyme assays

Carboxymethylcellulase (CMCase) activity was determined as the release of reducing sugars from carboxymethylcellulose (CMC) using a modification of a previously described method [17]. In each of two runs, determinations were made in duplicate. In the test solution, samples received 100 μl of the appropriate condensed tannin solution, 100 μl of the enzyme solution and 800 μl of substrate [1.5% (w/v) of CMC sodium salt, medium viscosity, (Sigma) in citrate-phosphate buffer, pH 6.5]. The tannin solutions were prepared by dissolving purified condensed tannin in 5% (w/v) methanol in citrate buffer solution (pH 6.5) to obtain concentrations of 0, 12.5, 25, 37.5 and 50 μg of condensed tannin ml^{-1} of reaction mixture. In the control solution, samples received 800 μl of citrate-phosphate buffer (pH 6.5) in place of the carbohydrate substrate solution. In both, treatment and control tubes, the reaction mixture was incubated for 30 min at 50 °C and release of reducing sugars determined using the method of Somogyi [18], reading absorbance of the copper-sugar-arsenomolybdate complex at 540 nm (FP-901 Chemistry Analyser, Lab Systems). Standard curves for released reducing sugars were obtained by reacting 100 μl of standard solution (containing 0, 0.25, 0.5, 1 or 2 mg of glucose ml^{-1} distilled water), 100 μl of 5% methanol in citrate buffer (pH 6.5) and 800 μl of either CMC (test solution) or buffer (control solution).

Xylanase activity was determined as the release of reducing sugars from soluble xylan (oat spelt xylan, Sigma X0627) using the protocol described for the CMCase assay, but with the following modifications. The substrate solution contained 6.25 mg ml^{-1} of soluble xylan [19] in citrate–phosphate buffer, pH 6.5. Standard solutions contained 0, 0.5, 1, 1.5, 2 and 3 mg of xylose ml^{-1} of distilled. In these assays, the condensed tannin concentrations used were 0, 3.125, 6.25, 12.5 and 25 μg of condensed tannin ml^{-1} of final reaction mixture.

The procedure used for determination of β-D-glucosidase activity was modified from Garcia-Campayo and Wood [20]. In each of two runs, determinations were made in triplicate in 1.5 ml Eppendorf tubes. Samples received 400 μl of citrate–phosphate buffer (pH 6.5), 50 μl of enzyme solution, 25 μl of substrate (25 mM of *p*-nitrophenyl-β-D-glycopyranoside, Sigma) and 25 μl of condensed tannin solution. Condensed tannin solutions provided 0, 12.5, 25, 50 and 100 μg of tannin ml^{-1} of reaction mixture. Enzyme blanks (400 μl of the assay buffer, 25 μl of substrate and 75 μl of water) and substrate blanks (400 μl of the assay buffer, 50 μl of enzyme and 50 μl of water) were included in each run. Samples were incubated for 1 h at 39 °C and the reaction terminated by the addition of 500 μl of NaOH–glycine buffer, pH 10.6. Aliquots (200 μl) of the resultant solutions were placed in 96-well micro-titre plates and their absorbance read (405 nm, BIO Kinetics Reader, Biotek Instruments, Luminar Technology Ltd.). Standard curves for *p*-nitrophenol were obtained by mixing 400 μl of the assay buffer, 50 μl of water and 50 μl of the standard solutions, which contained 0, 50, 100, 150, 200, and 300 mg of *p*-nitrophenol ml^{-1} in distilled water.

The activity of β-D-xylosidase was determined as described for β-D glucosidase, except that the substrate solution contained 25 mM of *p*-nitrophenyl-β-D-xylopyranoside (Sigma). The condensed tannin solution provided 0, 3.125, 6.25, 12.5, 25 and 50 μg of tannin ml^{-1} of reaction solution.

The kinetics of ferulate breakdown by a recombinant FAE was studied in two experiments. The first experiment evaluated the effect of reaction time between tannin and FAE on enzyme activity. Triplicate samples received 965 μl of sodium phosphate buffer (pH 5.0), 10 μl of the enzyme solution and 10 μl of a 95% ethanol solution containing 6 mg of *D. ovalifolium* condensed tannins ml^{-1}. Samples were then left to react at room temperature for 0, 1, 2, 4, 6 and 10 min after which, 25 μl of the substrate solution (5 mM ethyl-4-hydroxy-3-methoxy-cinnamate in 95% ethanol) was added. Upon mixing, the change in absorbance was read for two min at 334 nm (Philips PU 8720 UV/VIS scanning spectrophotometer).

The second experiment compared the effects of different types and concentrations of condensed tannin on FAE activity. Quadruplicate samples received 965 μl of sodium phosphate buffer, pH 5.0, 10 μl of the enzyme solution and 25 μl of substrate solution. After mixing, the change in absorbance at 334 nm was read for 45 s. Then, an aliquot (10 μl) of the appropriate tannin solution was added and after vigorous mixing, the change in absorbance at 334 nm was recorded for a further 60 s. In addition to condensed tannins from the tropical forages, condensed tannins from *L. corniculatus* were included in this experiment. At least five different concentrations were used for each tannin; all condensed tannin solutions were prepared in 95% ethanol. The rate of ferulate breakdown after addition of condensed tannin was expressed as a percentage of the rate observed in the first 45 s in the absence of the condensed tannin.

2.5. Activity of fibrolytic enzymes against plant cell walls from *Festuca arundinacea* as substrate

The inhibitory effect of condensed tannins added directly to reaction solutions containing particulate substrate (*F. arundinacea* plant cell walls) or bound to the surface of the cell walls was investigated. Two experiments were performed, each using a 1:1 (v/v) mixture of culture filtrate from CMCase and xylanase enriched cultures of *N. hurleyensis* as the enzyme solution; all other reagents were the same as used in the CMCase assay. The particulate substrate in these experiments was the lignin-free, primary cell walls of *F. arundinacea*. These were prepared from cells grown in culture [21]. The harvested cells were washed with distilled water and citrate buffer (pH 6.5) to remove sugars and other soluble components and then lyophilized. In the first experiment, 10 mg

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