International Journal of Biological Chemistry

ISSN 1819-155X

Academic Journals Inc.

www.academicjournals.com

International Journal of Biological Chemistry 6 (1): 31-36, 2012
ISSN 1819-155X / DOI: 10.3923/ijbc.2012.31.36

Reduced Tannin Content of Laccase-treated Cocoa (*Theobroma cacao*) Pod Husk

[1]C.A. Mensah, [1]N.A. Adamafio, [2]K. Amaning-Kwarteng and [3]F.K. Rodrigues
[1]Department of Biochemistry, Cell and Molecular Biology, University of Ghana, P.O. Box LG 54, Legon, Ghana
[2]Department of Animal Science, College of Agriculture and Consumer Sciences, University of Ghana, Ghana
[3]School of Applied Sciences, Central University College, Ghana

Corresponding Author: Naa Ayikailey Adamafio, Department of Biochemistry, Cell and Molecular Biology, University of Ghana, P.O. Box LG 54, Legon, Ghana

ABSTRACT

The high levels of anti-nutritional substances such as tannin and lignin in cocoa (*Theobroma cacao*) pod husk hinder its proper utilization by animals. The purpose of the present study was to isolate laccase from the residual substrate of oyster mushroom (*Pleurotus ostreatus*) in order to investigate its potential as a tool for the degradation of lignin and tannin in cocoa pod husk. Laccase (specific activity of 7.4 mU mg^{-1}) was extracted from spent sawdust and purified to electrophoretic homogeneity. The isolated enzyme oxidized 2,6-dimethoxyphenol optimally at pH 5.0 and showed maximum activity at 50°C. Treatment of cocoa pod husk with laccase for 40 min resulted in a 66% reduction in tannin content. Pretreatment of cocoa pod husk with laccase resulted in a higher rate of cellulose degradation *in vitro*. The enzyme was, however, unable to promote lignin depolymerization under the experimental paradigm employed. These findings provide an important opportunity for the isolation of laccase from the residual substrate of *Pleurotus ostreatus* on a commercial scale in Ghana. Treatment of cocoa pod husk with isolated extracellular laccase would reduce tannin levels considerably and thus, improve the nutritive value of cocoa pod husk-based animal feeds.

Key words: Laccase, cocoa pod husk, animal feed, tannin, *Pleurotus ostreatus*

INTRODUCTION

The poor degradability of cocoa (*Theobroma cacao*) pod husk (CPH) at high dietary concentrations has been attributed to the presence of anti-nutritional biomolecules, particularly tannins and lignin (Olubamiwa *et al*., 2002; Teguia and Beynen, 2005; Ocak, 2005; Alemawor *et al*., 2009). Owing to their high affinity for proteins, tannins bind to a variety of digestive enzymes, rendering them ineffective. As a consequence, tannins negatively affect the digestion process, reduce the efficiency of feed utilization and ultimately reduce the growth rate of animals (Mueller-Harvey, 2006; Waghorn, 2008). Additionally, tannins have an astringent, bitter taste that reduces palatability (Da Costa *et al*., 2008; Okuda and Ito, 2011). High dietary concentrations of lignin prevent enzymes from degrading structural polysaccharides thereby decreasing feed utilization and, ultimately, animal growth (Gomes *et al*., 2011).

Fermentation of cocoa pod husk with edible mushroom is reported to reduce tannin content significantly over a seven-week period (Alemawor *et al*., 2009). The development of a more rapid

but simple mushroom-based bioremediation procedure for eliminating tannins and possibly lignin, from cocoa pod husk would greatly facilitate the adoption of the biotransformation process as well as promote the utilization of the large quantities of husk discarded in the West African sub-region. Laccase (p-diphenol:oxygen oxidoreductase; EC 1.10.3.2) is an extracellular copper-containing polyphenol oxidase secreted by fungi such as *Pleurotus ostreatus*. Numerous potential applications of laccase in the elimination of environmental organopollutants and industrial contaminants have been investigated (Selinheimo *et al*., 2008; Djarwanto and Tachibana, 2010; Lettera *et al*., 2010; Das *et al*., 2011; Desai and Nityanand, 2011). Since the commercial cultivation of *Pleurotus ostreatus* strain EM-1 on sawdust is well established in Ghana, the present study was undertaken to isolate extracellular laccase from spent sawdust and investigate whether treatment of CPH with laccase would diminish lignin and tannin content significantly and thereby improve *in vitro* degradability.

MATERIALS AND METHODS

Bags of *Pleurotus ostreatus* cultivated on sawdust containing rice bran and lime were obtained locally. Husks of freshly harvested cocoa pods, from the Cocoa Research Institute of Ghana, were sun-dried and milled using a hammer mill with a screen size of 1 mm and stored at 4°C. Laccase from *Trametes versicolor* (0.72 U mg^{-1}), Cellulase from *Aspergillus niger* (45 mU mg^{-1}), 2,6-dimethoxyphenol (DMP), vanillin and 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS) were purchased from Fluka BioChemika, Germany. (±)-Catechin hydrate was obtained from Sigma-Aldrich Chemie, Germany.

Laccase preparation: Laccase secreted by *Pleurotus ostreatus* was isolated from sawdust and purified as previously described (Adamafio *et al*., 2008). A unit of laccase activity is defined as the amount of enzyme that oxidises one µmol of 2, 6-dimethoxyphenol (DMOP) per min at pH 5.0 and 45°C. Protein concentration was determined using the Folin-Lowry assay (Lowry *et al*., 1951).

Treatment of cocoa pod husk with laccase: Cocoa pod husk samples were treated with purified laccase (1:1.5 w/v). ABTS was added to give a final concentration of 0.5 µmol g^{-1} CPH. The mixture was saturated with oxygen and incubated at 50°C for 20 to 120 min. The incubation was terminated by boiling for 10 min. The contents were then oven-dried at 55°C to constant weight and stored at 4°C. For control samples, the enzyme was deactivated by 30 min of boiling prior to treatment.

Determination of tannin and lignin content: Tannin was determined using the vanillin/HCl assay (Price *et al*., 1978) and the detergent system of analysis was employed in estimating lignin content (Van Soest and Robertson, 1980).

***In vitro* degradability of cocoa pod husk:** The release of reducing sugars from untreated or laccase-treated CPH during exposure to exogenous cellulase was determined as previously described (Adamafio *et al*., 2009). Similar experiments were conducted using a commercial laccase (0.72 U mg^{-1}) from *Trametes versicolor* for purposes of comparison.

Statistical analysis: Analysis of variance (ANOVA) tests along with Least Significant Difference (LSD) post-hoc comparisons were conducted using Excel Data Analysis Statistical Software and Statgraphics-plus Software Programme (Version 3.0). The level of significance was set to $p<0.05$. Differences between means with $p<0.05$ were accepted as being statistically significant.

RESULTS

The specific activity of the partially purified laccase was 7.4 mU mg^{-1}. The estimated tannin content of untreated CPH was 5.1%. Treatment of CPH with laccase resulted in a significant ($p<0.05$) time-dependent reduction tannin content (Fig. 1). For instance, treatment of CPH with laccase for 20 min reduced tannin content by 56% while treatment for 40 min resulted in a 66% reduction in tannin content. Laccase had no effect on lignin content under the experimental conditions used in this study (Fig. 1).

Laccase-treated CPH released a slightly higher but statistically significant amount of reducing sugars than untreated CPH (Fig. 2). For example, pre-treatment of CPH with laccase for 60 min caused the subsequent cellulase-catalyzed release of 16.6 mg reducing sugars/g CPH, while untreated CPH yielded 10.6 mg reducing sugars g^{-1} CPH ($p<0.05$). The amount of reducing sugars produced by commercial-laccase treated CPH was significantly higher than that from laccase-treated CPH after 2 h of incubation with cellulase (Fig. 2).

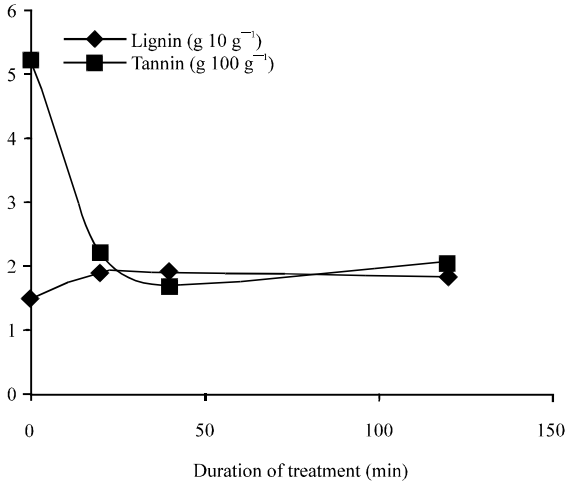

Fig. 1: Effect of laccase treatment on tannin and lignin content of CPH

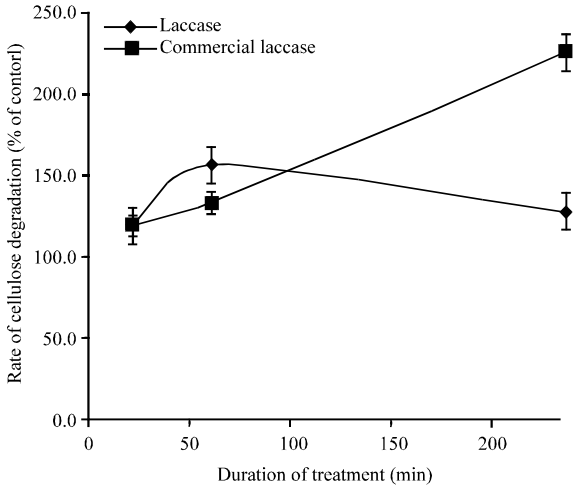

Fig. 2: Effect of laccase treatment on the rate of CPH cellulose degradation

DISCUSSION

The widely-accepted method for the isolation of laccase, involving the use of liquid cultures (Pointing *et al.*, 2000), is expensive, time-consuming and tedious. Since laccase is secreted during the cultivation of oyster mushroom and bags of spent sawdust are inexpensive and readily available in Ghana, the enzyme was extracted from residual substrate.

Treatment of CPH with the laccase preparation resulted in a significant ($p<0.05$) reduction in tannin content that was maximal (66%) after 40 min. The present findings are consistent with the reported effects of polyphenol oxidases on the astringency and tannin content of cocoa nibs (Brito *et al.*, 2002). The findings suggest that pre-treatment of crop residues with laccase would be an effective means of decreasing tannins levels and might ameliorate the deleterious effects associated with the ingestion of tannin-containing crop residues. Furthermore, it could form the basis of a rapid and effective procedure for eliminating tannins from CPH. Such a procedure would offer several advantages over other methods. It would drastically reduce the time and tedium associated with mushroom fermentation methods and would also avoid the problems related to the ingestion of residual alkali after treatment with wood ash and other kinds of alkali (Adamafio *et al.*, 2004; Salem *et al.*, 2005). This would be of considerable economic benefit to Ghana since it is estimated that the country generates more than five million metric tonnes of CPH annually.

Interestingly, although laccase is known to promote the depolymerization of lignin under conditions of oxygen availability (Ghosh *et al.*, 2008), the isolated laccase preparations used in the present study were found to be completely ineffective in degrading lignin. The numerous challenges associated with the determination of lignin are well-documented (Hindrichsen *et al.*, 2006) and it is quite plausible that the gravimetric procedure employed in the present study was not sufficiently sensitive to detect slight laccase-induced differences in content. Whether or not ligninolytic effects can be demonstrated at higher laccase concentrations remains to be determined. The transient nature of the modest increase in monosaccharide release following treatment with the isolated enzyme raises the possibility that responses to the laccase preparations were truncated or blunted by enzyme instability. This will be investigated in future studies.

In conclusion, treatment of CPH with extracellular laccase isolated from the growth substrate of *Pleurotus ostreatus* appears to be an effective means of reducing the tannin content of CPH and enhancing the degradability of its cellulose fraction *in vitro*. Moreover, the laccase-treatment would result in protein enrichment of CPH. Thus, the present findings hold much potential for the production tannin-free, protein-enriched CPH-based diets for livestock.

REFERENCES

Adamafio, N.A., E. Cooper-Aggrey, F.O. Quaye, J.K. Laary and J. Quaye, 2004. Effectiveness of corn stalk ash in reducing condensed tannin levels and improving *in vitro* enzymatic degradation of polysaccharides in crop residues. Ghana J. Sci., 44: 87-92.

Adamafio, N.A., K. Amaning-Kwarteng, F.K. Rodrigues and C.A. Mensah, 2008. A simple procedure for the isolation of laccase secreted by *Pleurotus ostreatus*. J. Ghana Sci. Assoc., 10: 78-84.

Adamafio, N.A., M. Obodai and B.B. Brimpong, 2009. Solid state fermentation of maize (*Zea mays*) cob by *Pleurotus ostreatus* strain EM-1: Biopolymer profiles and cellulose degradability. Int. J. Biol. Chem. Sci., 3: 1459-1466.

Alemawor, F., V.P. Dzogbefia, E.O.K. Oddoye and J.H. Oldham, 2009. Effect of *Pleurotus ostreatus* fermentation on cocoa pod husk composition: Influence of fermentation period and Mn^{2+} supplementation on the fermentation process. Afr. J. Biotechnol., 8: 1950-1958.

Brito, E.S., N.H.P. Garcia and A.C. Amancio, 2002. Effect of polyphenol oxidase (PPO) and air treatments on total phenol and tannin content of cocoa nibs. Cienc. Tecnol. Aliment., Vol. 22.

Da Costa, G., E. Lamy, F.S. Capela e, J. Andersen, B.E. Sales and A.V. Coelho, 2008. Salivary amylase induction by tannin-enriched diets as a possible countermeasure against tannins. J. Chem Ecol., 34: 376-387.

Das, N., S. Naskar, P. Chowdhury, B. Pasman, D. Adhikari 2011. Experimental evidence for presence of a growth regulating extracellular laccase in some *Pleurotus* species. Res. J. Microbiol., 6: 496-502.

Desai, S.S. and C. Nityanand, 2011. Microbial laccases and their applications: A review. Asian J. Biotechnol., 3: 98-124.

Djarwanto and S. Tachibana, 2010. Decomposition of lignin and holocellulose on *Acacia mangium* leaves and twigs by six fungal isolates from nature. Pak. J. Biol. Sci., 13: 604-610.

Ghosh, J.P., K.E. Taylor, J.K. Bewtra and N. Biswas, 2008. Laccase-catalyzed removal of 2,4-dimethylphenol from synthetic wastewater: Effect of polyethylene glycol and dissolved oxygen. Chemosphere, 71: 1709-1717.

Gomes, D.I., E. Detmann, S.V. Filho, R.S. Fukushima and M.A. de Souza *et al*., 2011. Evaluation of lignin contents in tropical forages using different analytical methods and their correlations with degradation of insoluble fiber. Anim. Feed Sci. Technol., 168: 206-222.

Hindrichsen, I.K., M. Kreuzer, J. Madsen and K.E. Bach Knudsen, 2006. Fiber and lignin analysis in concentrate, forage and feces: Detergent versus enzymatic-chemical method. J. Dairy Sci., 89: 2168-2176.

Lettera, V., A. Piscitelli, G. Leo, L. Birolo, C. Pezzella and G. Sannia, 2010. Identification of a new member of *Pleurotus ostreatus* laccase family from mature fruiting body. Fungal Biol., 114: 724-730.

Lowry, O.H., N.J. Rosebrough, A.L. Farr and R.J. Randall, 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem., 193: 265-275.

Mueller-Harvey, I., 2006. Unravelling the conundrum of tannins in animal nutrition and health. J. Sci. Food Agric., 86: 2010-2037.

Ocak, N., 2005. Rumen Degradability of dry matter and crude protein of fresh or dry lucerne and grass forages. J. Anim. Vet. Adv., 4: 324-328.

Okuda, T. and H. Ito, 2011. Tannins of constant structure in medicinal and food plants-hydrolyzable tannins and polyphehols related to tannin. Molecules, 16: 2191-2217.

Olubamiwa, O., A.R. Otun and O.G. Longe, 2002. Dietary inclusion rate of cocoa husk for starter cockerels. Int. J. Poult. Sci., 1: 133-135.

Pointing, S.B., E.B.G. Jones and L.P.P. Vrijmoed, 2000. Optimization of laccase production by *Pycnoporus sanguineus* in submerged liquid culture. Mycologia, 92: 139-144.

Price, M.L., S. van Scoyoc and L.G. Butler, 1978. A critical evaluation of the vanillin reaction as an assay for tannin in sorghum grain. J. Agric. Food Chem., 26: 1214-1218.

Salem, H.B., S. Abidi, H.P.S. Makkar and A. Nefzaou, 2005. Wood ash treatment a cost-effective way to deactivate tannins in *Acacia cyanophylla* Lindl. foliage and to improve digestion by Barbarine sheep. Anim. Feed Sci. Tech., 122: 93-108.

Selinheimo, E., P. Lampila, M.L. Mattinen and J. Buchert, 2008. Formation of protein-oligosaccharide conjugates by laccase and tyrosinase. J. Agric. Food Chem., 56: 3118-3128.

Teguia, A. and A.C. Beynen, 2005. Alternative feedstuffs for broilers in Cameroon. Livestock Res. Rural Dev., Vol. 17.

Van Soest, P.J. and J.B. Robertson, 1980. Systems of Analysis for Evaluating Fibrous Feeds. In: Standardisation of Analytical Methodology for Feeds, Pigden, W.J., C.C. Bolch and N. Graham (Eds.). International Development Research Center, Ottawa, Canada, ISBN-10:100889362173, pp: 46-60.

Waghorn, G., 2008. Beneficial and detrimental effects of dietary condensed tannins for sustainable sheep and goat production-Progress and challenges. Anim. Feed Sci. Technol., 147: 116-139.