THE ACCURACY AND USABILITY OF THE RADIAL GEL DIFFUSION ASSAY AND A DYE-RELEASE TECHNIQUE FOR DETERMINATION OF β-GLUCANASE IN FEED.

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Summary

Two enzyme assays, the radial diffusion and the dye-release methods, were modified and tested for their suitability in measuring β-glucanase activity in poultry feed. Enzymes A and B were incorporated into a barley-based broiler starter diet at three levels (500, 1000 and 2000 g/t). The measured β-glucanase activities were 35 U/g and 49 U/g for Enzymes A and B, respectively. A comparison of enzyme recovery rates from samples containing known amounts of the enzyme and from a buffer solution containing the same amount of the enzyme revealed that a fixed percentage of the enzyme bound to feed components in a dose responsive manner. Thus, enzyme recovery rates for the feed samples were not complete and varied between 66% and 97% for the radial diffusion assay and between 76% and 91% for the dye-release assay. The two assays largely agreed with each other although at low enzyme concentrations, the dye-release assays gave higher recovery rates with lower standard errors. It may be concluded that both assays are useful in determining β-glucanase activity in feed and digesta samples, but the radial diffusion assay is cheaper to perform.

I. INTRODUCTION

Application of feed enzymes has expanded into various sectors of the feed and livestock industries. This has placed a more urgent need for rapid and reliable assays for determination of enzyme activities in feed. Currently, the dye-release method for measuring xylanase activity in feed is widely used in the pig and poultry industries around the world, but there is not an equivalent assay for β-glucanase specifically developed for the feed industry. A number of other techniques, including the viscometric technique and the radial gel diffusion method, have been used to determine the activities of non-starch polysaccharide-degrading enzymes (Rotter et al., 1990; McCleary, 1995). The viscometric technique is used to determine the effect of glucanases on reducing viscosity using pure substrates, such as soluble arabinoxylans, β-glucans and pectins as standards, whereas the radial gel diffusion assay is based on the diffusion of enzyme-containing solutions from wells through a gel medium containing a substrate, e.g., β-glucan, stained with congo red. As the dyed substrate is depolymerised, Congo red which has a strong affinity to polysaccharides containing contiguous β(1→4) linked D-glucopyranosyl residues (Wood, 1981), is released and diffuses outward in a radial manner leaving a circular clear zone (Walsh et al., 1995). A linear relationship between the diameter of the clear zone and log of the enzyme concentration provides the basis for activity determination. A β-glucanase assay has been developed and used routinely for the determination of β-glucanase activity in malt (McCleary and Shameer, 1987), but its suitability for use in the feed industry has not been fully evaluated.

This study investigates the suitability of the radial gel diffusion assay for determining β-glucanase activity in feed in comparison with the dye-release assay.

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II. MATERIALS AND METHODS

(a) Preparation of feed samples and enzyme extracts

Two β-glucanase sources were added at dosages of 0.5, 1.0 and 2.0 g per kg of broiler feed. Enzyme A was a dry commercial β-glucanase product (Kemin Industries, Singapore), whereas Enzyme B was a liquid lichenase of high purity (Megazyme, Co. Wicklow, Ireland). The diets were cold pelleted at 60°C. The enzymes in the diets were extracted from 20.0 g of sample with 200 ml of phosphate buffer. After shaking, the flasks were allowed to stand for 10 min before aliquots of supernatant buffer were filtered through 0.2 µm filter discs.

(b) Standard curves

The enzyme standards were prepared by adding 0, 0.025, 0.050, 0.075 and 0.100 % (w/w) of the enzyme to 10.0 g of untreated (control) feed. The enzymes were extracted with 100 ml of 0.01 M phosphate buffer (pH 6.0) with continuous orbital shaking for 15 min.

(c) Radial Diffusion Assay

Agar plates were prepared by dissolving 0.03 % β-glucan from barley (Megazyme) in boiling 0.1 M phosphate buffer (pH 6.0). Congo red and bacteriological agar were then added at 0.007 and 1.0 %, respectively, in succession and boiled until a clear solution was obtained. Approximately 25 ml of the agar solution were poured into Petri dishes (9 cm diameter). Small wells of 6 mm in diameter were made in the plates and 70 µl of feed extract and enzyme standards were loaded. The plates were then incubated at 37°C for 16 h. The diffusion radius was then measured and enzyme activities calculated in relation to the standard controls.

(d) Dye-release assay

The assay developed was modified from the malt β-glucanase assay procedure provided by Megazyme. To each tube containing 0.5 ml of Azo-barley glucan substrate, 1.0 ml of feed extract (pH 6.0) was added and mixed before incubation for 10 min at 37°C. Then 3.0 ml of a precipitant solution (made up of 40 g sodium acetate and 4.0 g of zinc acetate in 150 ml water) were added. The pH was adjusted to 5.0 with concentrated HCl and volume adjusted to 200 ml. This solution was finally mixed with 800 ml of methyl cellosolve (2-methoxyethanol) and left to stand for 5 min, and then centrifuged for 5 min at 1,700 g. The absorbance of the supernatant was read at 590 nm and β-glucanase activity calculated according to McCleary and Shameer (1987).

III. RESULTS

(a) Standard curves

Standard curves relating the “clear zone”, as a result of enzymatic hydrolysis, and the logarithmic function of enzyme concentration were constructed with filtered and non-filtered feed extract supernatants (Figures 1a,b). A third standard curve was constructed with the same enzyme stock solution that was diluted in buffer (i.e., without the addition of feed). The unfiltered extract resulted in higher hydrolysis rates than the filtered extracts. The enzyme
solution diluted with buffer gave the highest hydrolytic rate, indicating some of the enzymes binding to feed particles.

Figure 1. Standard curves for Enzyme A (Figure 1a, left) and Enzyme B (Figure 1b, right), showing the logarithmic function of enzyme concentrations with areas of "clear zone". Three curves display standard curves produced from filtered feed samples (♦), unfiltered samples (■), and no feed controls (▲).

(b) Enzyme recovery rates

The recovery rates for Enzymes A and B were above 50% of the expected values in all cases (Table 1). For most doses for both enzymes, the recovery rates obtained using the two assays were similar, although the dye-release assay gave numerically higher recovery rates when the level of enzymes in the feed was below 1000 g/t (Table 1).

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Expected (g/t)</th>
<th>Radial Diffusion % recovery</th>
<th>Dye-Release g/t % recovery</th>
<th>Assay Difference %</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>500</td>
<td>328 ± 64 65.6</td>
<td>389 ± 62 77.8</td>
<td>-15.7</td>
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<tr>
<td>A</td>
<td>1000</td>
<td>766 ± 132 76.6</td>
<td>870 ± 72 87.0</td>
<td>-12.0</td>
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<tr>
<td></td>
<td>2000</td>
<td>1810 ± 173 90.5</td>
<td>1733 ± 14 86.7</td>
<td>4.4</td>
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<tr>
<td></td>
<td>500</td>
<td>344 ± 64 68.8</td>
<td>378 ± 30 75.6</td>
<td>-9.0</td>
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<tr>
<td>B</td>
<td>1000</td>
<td>970 ± 171 97.0</td>
<td>913 ± 179 91.3</td>
<td>6.2</td>
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<tr>
<td></td>
<td>2000</td>
<td>1701 ± 64 85.1</td>
<td>1638 ± 22 81.9</td>
<td>3.9</td>
</tr>
</tbody>
</table>
IV. DISCUSSION

Little information is available on the survival rate of enzymes during processing (mixing, steam-conditioning, pelleting and storage) and in the digestive system (gastric acid, proteolytic hydrolysis and pH) before reaching the small intestine. Thus, the extent of "over-dosing" of supplementary enzymes, a common practice in the industry to ensure efficacious responses, is unknown. The current study assessed the effectiveness of the radial gel diffusion assay and a modified dye-release assay in determining the $\beta$-glucanase activity in feed, and possibility of developing them as a research tool for measuring enzyme activities in digesta samples.

The current study explored the possibility of using unfiltered feed samples for determination of enzyme activities in order to simplify the assay procedure further. The data from the standard curves relating the zone of hydrolysis and log of enzyme concentration for the radial diffusion assay demonstrated clearly that (a) filtering did not make any difference for the sensitivity of the assay, and (b) a portion of the exogenous enzyme was bound to feed components and was not extractable for analysis. The latter finding is consistent with McCleary, (1995) work, with the exception of the standard curves for the concentrations tested in this investigation that were almost parallel to the standard curve constructed without the presence of feed (Figure 1). This would suggest that the feed bound a fixed percentage rather than a fixed amount of the exogenous enzymes. It is, however, not known whether this linear relationship would hold over a wide range of enzyme concentrations in the feed. The results also showed that a percentage of the exogenous enzymes were bound to the filter disc membranes, highlighting the importance of constructing appropriate standard curves in order to obtain accurate recovery results.

The majority of the recovery results from the dye release assay correlated well with those from the radial gel diffusion assay (Table 1) although there were differences of up to 15% between the assays when the level of enzyme was low in the feed. Both assays are easy to conduct and do not require a large amount of samples. The dye-release assay produced less error, most likely the result of less subjectivity in measurement as compared to the radial gel diffusion assay. Another advantage of the dye-release assay is the speed with which results could be obtained. However, a drawback of this assay is the cost of the chromogenic substrate.

Radial gel diffusion and dye-release assays were developed and successfully used in the determination of supplementary $\beta$-glucanase in feed. Further research is required to refine the methods to improve their consistency.

REFERENCES