ENZYMES IN FARM ANIMAL NUTRITION

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Analysis of Feed Enzymes

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Introduction

Enzymes are added to animal feed to increase its digestibility, to remove anti-nutritional factors, to improve the availability of components, and for environmental reasons (Campbell and Bedford, 1992; Walsh *et al.*, 1993). A wide variety of carbohydrase, protease, phytase and lipase enzymes find use in animal feeds. In monogastric diets, enzyme activity must be sufficiently high to allow for the relatively short transit time. Also, the enzyme employed must be able to resist unfavourable conditions that may be experienced in feed preparation (e.g. extrusion and pelleting) and that exist in the gastrointestinal tract. Measurement of trace levels of enzymes in animal feed mixtures is difficult. Independent of the enzyme studied, many of the problems experienced are similar; namely, low levels of activity, extraction problems, inactivation during feed preparation, non-specific binding to other feed components and inhibition by specific feed-derived inhibitors, e.g. specific xylanase inhibitors in wheat flour (Debyser *et al.*, 1999).

In this chapter, some of the procedures used to assay for β-glucanase, β-xylanase, α-amylase, α-galactosidase, phytase and *endo*-protease enzymes will be described. In particular, some of the problems and limitations of current assay procedures will be discussed.

β-Glucanase

The anti-nutritional properties of β-glucans (1,3 : 1,4-β-D-glucans, mixed-linkage β-glucans) have been known for many years (Aastrup, 1979). In animal feeds the major source of β-glucan is barley grain. Although levels are also high in oats, these are rarely fed to chickens and pigs. The anti-nutritional properties of β-glucan are attributed to their viscosity-inducing properties, which significantly affect the rate of movement of barley-based diets through the digestive tract of chickens, and reduce the rate of nutrient absorption. This problem is effectively 'dissolved' by the judicious use of β-glucan-degrading enzymes.

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A wide range of enzymes are active on β-glucan and these include the *endo*-acting fungal cellulases [*endo*-1,4-β-D-glucanase (EC 3.2.1.4)] and the bacterial β-glucanase [lichenase; *endo*-1,3 : 1,4-β-D-glucanase (EC 3.2.1.73)]. Both groups of enzymes cleave within the main chain of mixed-linkage β-glucan, although their point of action is different (Fig. 4.1). Fungal cellulases also hydrolyse cellulose (1,4-β-D-glucan) in an *endo*-hydrolytic pattern, but the bacterial 1,3 : 1,4-β-glucanases have no action on cellulose. This point of difference is important in designing specific substrates for the assay of these groups of enzymes.

A range of substrates and assay procedures is available for the measurement of bacterial β-glucanase and cellulase enzymes, some of which are listed in Table 4.1. Bacterial 1,3 : 1,4-β-D-glucanase is assayed using substrates based on a 1,3 : 1,4-β-D-glucan polysaccharide, i.e. oat or barley β-glucan or lichenan. Procedures for the purification of barley and oat β-glucan have been developed and are reported in the literature (McCleary, 1988), and high purity polysaccharides are commercially available. However, the purification of lichenan is much more difficult. Lichenan is purified from Icelandic moss, but it occurs together with isolichenan (Chandra *et al.*, 1957), from which it is hard to separate. Other glucose-containing polysaccharides (starch and cellulose) are absent from purified lichenan.

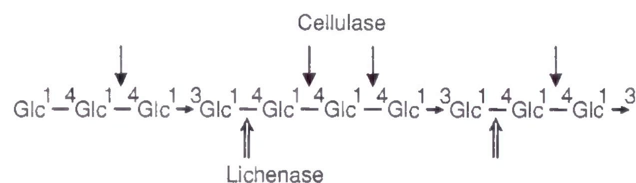

Fig. 4.1. Schematic representation of the mode of action of cellulase and 1,3 : 1,4-β-glucanase on mixed-linkage β-glucan.

Table 4.1. Substrates for the assay of cellulase and β-glucanase.

Substrate	Nature	Assay procedure
Cellulase		
Barley β-glucan	Soluble	Reducing-sugar or viscometric
Lichenan	Soluble	Reducing-sugar or gel plate
CMC-7M	Soluble	Reducing-sugar or viscometric
CMC-4M	Soluble/gel	Reducing-sugar
Azo-CMC	Soluble	Chromogenic substrate
Azo-barley glucan	Soluble	Chromogenic substrate
Cellazyme C tablets	Gel particles	Chromogenic substrate
Cellazyme T tablets (tamarind xyloglucan)	Gel particles	Chromogenic substrate
Beta-Glucazyme tablets	Gel particles	Chromogenic substrate
1,3 : 1,4-β-Glucanase		
Barley β-glucan	Soluble	Reducing-sugar or viscometric
Azo-barley glucan	Soluble	Chromogenic substrate
Beta-Glucazyme tablets	Gel particles	Chromogenic substrate

Reducing-sugar methods

Pure β-glucan (barley or oat) can be used directly to assay cellulase or β-glucanase employing either a reducing-sugar or viscometric assay procedure. Of the reducing-sugar procedures available, the only commonly used method that gives a stoichiometric colour response with homologous oligosaccharides of varying degrees of polymerization (DP) is the Nelson/Somogyi procedure (Somogyi, 1960). Consequently, it is the only method that gives a true measure of glycosidic bonds cleaved, and thus of enzyme activity. For this reason, this is the method of choice for use as a reference method against which all other assay procedures can be compared and standardized. Details of a suggested format for the assay of β-glucanase using barley β-glucan and the Nelson/Somogyi reducing-sugar procedure are given in Appendix 4.1.

Several cellulose-based substrates are available for the assay of *endo*-1, 4-β-glucanase (cellulase). These substrates range from highly crystalline cotton cellulose to amorphous cellulose and lightly or heavily substituted chemical derivatives such as carboxymethyl-cellulose 4M (CMC-4M) and CMC-7M. CMC-4M is a medium viscosity (300–600 cP at 2%, 25°C) cellulose with a degree of substitution with carboxymethyl groups of 0.4 (i.e. over ten sugar residues, only four of the 30 available hydroxyl groups are substituted by carboxymethyl groups). CMC-7M is completely soluble, whereas CMC-4M at 1% concentration in water is somewhere between a solution and a colloidal suspension. Many cellulase enzymes hydrolyse CMC-7M at the same rate as CMC-4M, but for some the rate is substantially lower. Consequentially, CMC-4M is the substrate of choice. Cellulase enzymes act on other polysaccharide substrates, such as mixed-linkage β-glucan and tamarind-seed xyloglucan (which is composed of a 1,4-β-D-glucan backbone, to which D-xylose is attached in a relatively regular manner, some of which is further substituted by D-galactose). Both of these polysaccharides serve as the base for excellent dyed substrates for the assay of cellulases (Table 4.1).

Traditionally, cellulase activity has been standardized using a reducing-sugar assay with either CMC-7M or barley β-glucan as substrate. In most cases the dinitrosalicyclic acid (DNSA) reducing-sugar method (Bailey, 1988) was used with cellobiose as the standard. The DNSA method does not give a stoichiometric colour response with homologous oligosaccharides of varying degrees of polymerization; thus it is not ideal as a reference method. The Nelson/Somogyi reducing-sugar method (Somogyi, 1960) is preferable. Also, since some cellulase enzymes hydrolyse CMC-4M more rapidly than CMC-7M, then CMC-4M or barley β-glucan are the substrates of choice.

Viscometric methods

The Nelson/Somogyi reducing-sugar procedure (Somogyi, 1960) is an excellent method for the assay and standardization of relatively pure β-glucanase preparations, i.e. preparations essentially devoid of other enzyme activities active on the substrate,

and for preparations containing low levels of reducing-sugars (which interfere with the assay). However, the method cannot be applied to the measurement of β-glucanase in animal feed mixtures. For such materials, other procedures need to be employed, such as viscometric methods and methods based on the use of dyed polysaccharide substrates. Both procedures specifically measure *endo*-hydrolase activity and can be used in the presence of high levels of reducing sugars.

In industry, the most commonly used viscometric assay for polysaccharide *endo*-hydrolase activity is the Institute of Brewing procedure for the measurement of β-glucanase in malt (Bathgate, 1979). For reproducibility, accuracy and reliability, a β-glucan preparation of high purity and a defined viscosity range is essential. In principle, the enzyme preparation is mixed with buffered β-glucan solution (10 mg ml^{-1}) under controlled temperature conditions. The viscosity (as the time to flow between two marked points on a standardized type C U-tube viscometer) is measured at various time intervals after the addition of the enzyme to the substrate. The inverse reciprocal viscosity is calculated and plotted against time of incubation (see Appendix 4.2). The slope of the curve is a direct measure of enzyme activity. An alternative substrate is CMC-7M. This is completely soluble in water and is rapidly hydrolysed by some *endo*-cellulases. A procedure for the measurement of β-glucanase or xylanase in animal feeds, based on the Institute of Brewing IRVU method, is given in Appendix 4.2.

Agar plate diffusion procedures

Several semi-quantitative procedures have been developed for the assay of enzyme activity based on the rate and degree of diffusion of the enzyme through agar plates. The enzyme is applied in a central well and diffuses through agar containing a specific substrate. The level of activity is then monitored by staining the plate with a dye specific for the particular polysaccharide substrate. In one such procedure, Walsh *et al.* (1995) detected β-glucanase using an agar plate impregnated with lichenan, and activity was detected by staining with Congo Red. These procedures do work, but at best are semi-quantitative.

Soluble, dye-labelled substrates

The two general types of dye-labelled substrates available for the assay of cellulase and β-glucanase are soluble dye-labelled substrates and insoluble dyed substrates. The soluble dyed substrates include azo-CM-cellulose (azo-CMC) and azo-barley glucan (McCleary and Shameer, 1987). These substrates find widespread application in the assay of β-glucanase in feeds (Rotter *et al.*, 1990; Cosson *et al.*, 1999). In Fig. 4.2, a standard curve relating enzyme activity to colour released on hydrolysis of azo-CMC (CMC-4M dyed with Remazolbrilliant Blue R dye) is shown. The curves obtained for the two unfractionated commercial enzyme preparations and for a purified cellulase from a *Trichoderma* sp. are relatively similar. Differences can be attributed

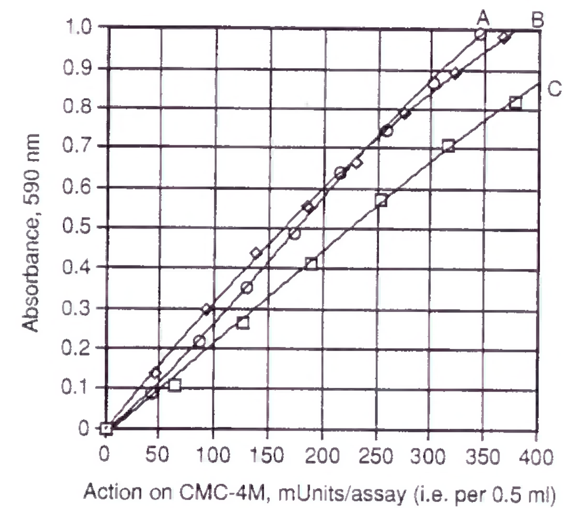

Fig. 4.2. Standard curves for the action of cellulase preparations on azo-CMC (lot 60601): (A) a crude *Trichoderma viride* preparation; (B) a crude *T. longibrachiatium* preparation; (C) a pure cellulase from a *T. longibrachiatium* preparation.

to the range of activities in the crude preparations, as well as to subtle differences in the action patterns of the individual *endo*-cellulase enzyme components. This procedure has been adopted by the UK silage industry for the standardization of cellulase activity in silage additive preparations. The major limitation in the application of this procedure to measurement of cellulase activity in animal feed preparations is the lack of sensitivity. A second concern is the fact that the base substrate is CM-cellulose rather than β-glucan, which is the target substrate in animal feeds. The action of pure *Trichoderma* sp. cellulase on azo-barley glucan (barley glucan dyed with Remazolbrilliant Blue R dye) is shown in Fig. 4.3. The sensitivity with azo-barley glucan is about three times that with azo-CMC, but unfortunately is still not adequate for the measurement of trace levels of cellulase in animal feeds. However, it has been found that action on azo-barley glucan is a good predictor of *in vivo* response to cellulase supplementation of barley-based diets in young chickens (Rotter *et al.*, 1990).

Insoluble dye-labelled substrates

Insoluble dyed substrates are prepared by dyeing and cross-linking soluble polysaccharides to give dyed cross-linked polysaccharide gel matrices. Such substrates are readily hydrolysed and include AZCL-HE-cellulose (Cellazyme C tablets), AZCL-β-glucan (Beta-Glucazyme tablets) and AZCL-xyloglucan (Cellazyme T tablets). Assays based on the use of these substrates are three to ten times more sensitive than assays using azo-barley glucan or azo-CMC. In Fig. 4.4, standard curves for

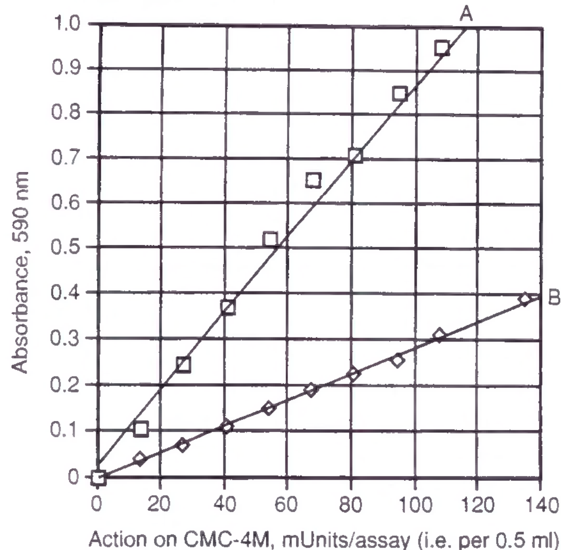

Fig. 4.3. Standard curve relating the activity of a pure *T. longibrachiatium* cellulase on CMC-4M (Nelson/Somogyi assay) to action on: (A) azo-barley glucan (lot 60602) (using EGME/zinc acetate-based precipitant) and (B) azo-CMC (lot 60601) (using ethanol/zinc acetate-based precipitant).

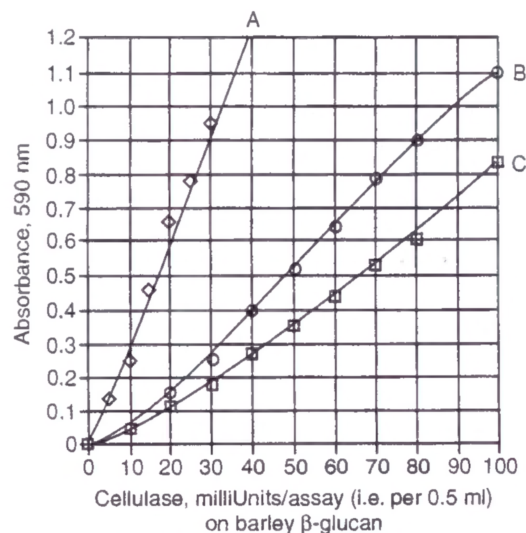

Fig. 4.4. Standard curves relating the activity of a pure *T. longibrachiatium* cellulase on barley β-glucan (Nelson/Somogyi assay) to activity on: (A) Cellazyme T tablets (lot 70801); (B) Beta-Glucazyme tablets (lot 50901); and (C) Cellazyme C tablets (lot 80201).

Trichoderma sp. cellulase on dyed cross-linked HE-cellulose (Cellazyme C), dyed cross-linked β-glucan (Beta-Glucazyme) and dyed cross-linked tamarind xyloglucan (Cellazyme T tablets) are compared. It is evident that, of the substrates compared, the Cellazyme T tablets give the greatest sensitivity, and thus are the substrate of choice for the detection and measurement of trace levels of cellulase in animal feed. The reason for this greater sensitivity must be due to the regular distribution of xylose residues along the cellulose backbone of the polysaccharide, compared with the irregular to random distribution of hydroxypropyl residues in HE-cellulose.

Measurement of cellulase and β-glucanase enzymes in feed is complicated by several factors, including inactivation of the enzyme during feed pelleting, adsorption of the enzymes to feed components, competition with endogenous substrate and possible enzyme inhibitors. These same problems are experienced in the assay of xylanase and protease enzymes in feed.

Endo-1,4-β-D-Xylanase (Xylanase)

The major endosperm cell-wall polysaccharide in wheat and rye grain is arabinoxylan, which represents about 2–5% of the grain weight. About two-thirds of wheat-flour arabinoxylan is water insoluble (Amado and Neukom, 1985). However, both the soluble and the insoluble components have high water-absorbing properties. Both can absorb about ten times their weight of water (Kulp, 1968). Consequently, arabinoxylans in feed cause problems similar to those experienced with β-glucans (Annison and Choct, 1991). The development and application of a range of xylanase enzymes have resolved these problems. Measurement of xylanase in feeds is complicated by the fact that the levels added are very low, and this enzyme can be lost through inactivation during pelleting, adsorption to feed components or inactivation by specific xylanase inhibitors (Debyser *et al.*, 1999).

Several assay procedures have been developed for the assay of xylanase, and these include reducing-sugar assays, viscometric assays, and assays based on the use of chromogenic substrates (Table 4.2).

Reducing-sugar and viscometric assays

Pure wheat arabinoxylan forms the basis of both reducing-sugar and viscometric assays for xylanase. Reducing sugar assays are not specific and cannot be used for the measurement of xylanase in trace levels in feed mixtures. However, the Nelson/Somogyi reducing-sugar procedure (Somogyi, 1960) is the method of choice for the standardization of pure enzyme preparations. These enzyme preparations can then be used to standardize other assay procedures that use less defined substrates (e.g. the dyed xylan and arabinoxylan substrates) and assay procedures that are expressed in non-conventional units (e.g. viscometric assays). Viscometric assays are used in some laboratories, and the substrate generally employed is wheat arabinoxylan (20–30 cSt). Other xylan substrates (from oat and birchwood) are available, but the

within a period of linear release with time. One unit of phytase activity is defined as the amount of enzyme required to release 1 μmol of orthophosphate from phytic acid under standard assay conditions (pH 5.5, 37°C) in 1 min. However, for general acceptance of phytase enzyme as a feed additive, there is a need for validation of this type of assay. To satisfy this need, a major international evaluation of an assay format has been performed under the auspices of AOAC International. The results of these studies are currently under evaluation (P. Randsdorp, personal communication, 1999).

endo-Protease

Several substrates and assay procedures are available for the measurement of protease. Many of those based on the use of native proteins such as casein, albumin and haemoglobin do not distinguish between *endo*- and *exo*-protease (peptidase) activity. Assays based on trichloroacetic acid (TCA) precipitation of non-hydrolysed substrate and measurement of the absorption (235 nm) of the supernatant solution on centrifugation (Kunitz, 1947; AACC, 1985) are likely to be more specific than procedures that measure the release of free amino groups (Lin *et al.*, 1969). However, even TCA precipitation methods are not specific. A greater specificity can be obtained with dye-labelled proteins (Charney and Tomarelli, 1947), or dye-labelled and cross-linked proteins. Action of *exo*-acting peptidases on these substrates will be hindered or stopped by the dye molecule, or at the point of cross-linking.

Several dyed protein substrates are commercially available, including albumin, casein and collagen dyed with sulphanilic acid (azo-albumin, azo-casein and azo-collagen), and collagen dyed with Remazolbrilliant Blue (Hide powder azure). These are useful substrates and form the basis of simple assay procedures, but the quality of commercially available substrates varies significantly, limiting their value as analytical reagents. Standard curves for Subtilisin A on two commercially available azo-casein substrates are shown in Fig. 4.10 and regression equations for several proteases on one of the substrates is shown in Table 4.3 (McCleary and Monaghan, 1999). It is evident that one of the preparations (ex. Megazyme) is more useful than the other, with better sensitivity and linearity of the standard curve. This preparation is useful in the assay of industrial enzyme preparations, but problems are experienced in the measurement of enzymes in feed. Whether this is due to binding of the enzyme to the feed, inhibition by endogenous protease inhibitors or competition by other protein substrates is not clear.

An alternative *endo*-protease substrate, and one that finds considerable use, is cowhide azure (basically collagen dyed with Remazolbrilliant Blue). This substrate is very fibrous and difficult to weigh accurately. Consequently, an alternative collagen-based substrate was prepared by dyeing and cross-linking collagen from the swim bladders of fish. The structure of this collagen is very similar to that in cowhide. The major difference in the final product is that the new product from fish collagen (AZCL-collagen) can be milled to a fine powder and incorporated into tablets (Protazyme OL). The suitability of this material as a general or selective substrate was

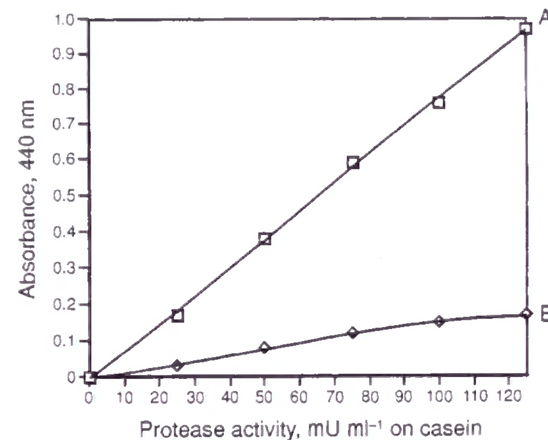

Fig. 4.10. Standard curve relating the activity of Subtilisin A on casein to action on two commercially available azo-casein preparations: (A) Megazyme (lot 81001); (B) Sigma Chemical Co. (lot 74H7165).

Table 4.3. Action of protease enzymes on azo-casein (lot 81001).

Enzyme	Protease (mU ml^{-1})[a,b] (R = 0.99)	Linear absorbance range
Papain (from *Papaya latex*)	270 × Abs. (440 nm) + 7	0.1–1.0
Bromelain (from pineapple stem)	460 × Abs. (440 nm) – 13	0.1–0.9
Ficin (from figs)	190 × Abs. (440 nm) + 3	0.1–1.1
Subtilisin A (from *Bacillus licheniformis*)	129 × Abs. (440 nm) + 4	0.1–1.0
Bacterial protease (from *Bacillus subtilis*)	250 × Abs. (440 nm) – 8	0.1–1.0
Proteinase K (from *Tritirachium album*)	140 × Abs. (440 nm) – 4	0.1–1.0
Fungal protease (*A. niger*, from Sigma Chemical Co.)	146 × Abs. (440 nm) – 4	0.1–1.0

[a]One protease unit is defined as the amount of enzyme that will produce the equivalant of 1 μmol tyrosine min^{-1} from soluble casein at pH 7.0 and at 40°C.
[b]Abs. = absorbance.

determined by comparing the activity of a wide range of proteases on this substrate to a similar material prepared from casein (AZCL-casein; Protazyme AK). The conclusion derived from this study was that a similar relative rate of hydrolysis of the two substrates was obtained for every protease tested. Consequently, AZCL-collagen had no particular advantage over AZCL-casein, and since the standard curves obtained with AZCL-casein (Protazyme AK tablets) were consistently more linear, we consider that this is the substrate of choice.

Assays with Protazyme AK substrate are four to five times more sensitive than those with Azo-casein, and the standard curves obtained for all of the proteases studied are linear (Table 4.4). However, the substrate tablets do not hydrate as rapidly as do tablets containing dyed and cross-linked polysaccharide substrates. Thus, for good reproducibility, the tube contents should be stirred during the

Unit definition

One inverse reciprocal viscosity unit (IRVU) is the increase in reciprocal viscosity h^{-1} ml^{-1} (or g^{-1}) of enzyme (or plant material), under standard assay conditions.

Preparation of β-glucan or arabinoxylan substrate

1. Barley β-glucan (1 g, pure, 20–30 cSt) or wheat arabinoxylan (1 g, pure, 20–30 cSt) is accurately weighed into a 120 ml dry pyrex beaker.
2. The sample is wetted with 6 ml of 95% ethanol and then with 80 ml of cold water.
3. A magnetic stirrer bar is added to the beaker and the beaker is placed on a magnetic stirrer-hotplate and heated at a setting of 120°C with vigorous stirring. The beaker is loosely covered with aluminium foil and stirred and heated for about 15 min.
4. The polysaccharide should completely dissolve. If dissolution is not complete, continue stirring for a further 30 min with the heating turned off.
5. Add 10 ml of acetate buffer (1 M, pH 4.6) or 20 ml of phosphate buffer (0.5 M, pH 6.0) and adjust the pH to the desired value.
6. Adjust the volume to 100 ml and store in a well-sealed glass bottle. Prevent microbial contamination by adding a few drops of toluene. The solution can be stored at room temperature for several weeks.

Enzyme extraction and dilution

1. Plant samples are milled to pass through a 0.5 mm sieve on a Retsch centrifugal mill (or similar).
2. Accurately weigh 1.0 g of flour or powdered enzyme preparation into a 250 ml Erlemeyer flask and add 100 ml of appropriate extraction buffer; stir and extract over 15 min at room temperature.
3. Filter an aliquot of the slurry through a Whatman No. 1 filter circle, or centrifuge at 3000 rpm for 10 min. Store the filtrate in an ice bath.
4. Dilute the filtrate (or supernatant) with the appropriate buffer (by sequential dilution of 1 ml to 10 ml with dilution buffer) to give an enzyme concentration suitable for assay (i.e. a slope value '*A*' of 0.06 to 0.60).
5. Prepare liquid enzyme samples by dilution of 1.0 ml to 100 ml with appropriate buffer. This solution is further diluted as for extracts of dry samples.

Viscometric assay of activity

1. Pre-equilibrate the enzyme preparation at 40°C for 5 min and then pipette 1 ml of this solution into 12 ml of pre-equilibrated, buffered substrate solution (wheat arabinoxylan or β-glucan; 1% w/v) in a type C U-tube viscometer (in a water bath at 40°C), and mix the contents by blowing air into the viscometer tube. Immediately start a stop clock and leave this running throughout the entire assay to record incubation time (in minutes).
2. Using a second stop clock, take five falling time readings (in seconds) over a period of approximately 30 min. Take the time for each reading as the elapsed time from mixing the enzyme/substrate solutions to the mean of the falling time.
3. The viscosity (n) of the reaction digest is proportional to the falling number according to the following equation:

$$n_{digest} = (t_{digest} - t_{solvent})/t_{solvent}$$

where n_{digest} = specific viscosity of the digest; t_{digest} = falling time in seconds of the digest; and $t_{solvent}$ = falling time in seconds of the buffer (100 mM sodium acetate).

Calculations

The reciprocal viscosity ($1/n$) (Table 4.5) is calculated and plotted against incubation time (in minutes) (Fig. 4.11). The slope (A) is determined from the linear graph in terms of increase in reciprocal viscosity per hour.

$$\text{IRV units} = A \times 100 \times Dilution$$

where A = slope from graph in terms of increase in reciprocal viscosity per hour; *100* = 1 g of original enzyme preparation extracted with 100 ml of buffer (100 mM), or 1 ml of liquid enzyme preparation diluted to 100 ml with extraction/dilution buffer; and *Dilution* = further dilution of the extract or diluted liquid enzyme concentrate required to get an appropriate activity for assay.

Appendix 4.3 Extraction and Assay of Xylanase Enzymes in Animal Feeds

Example: *Trichoderma* sp. xylanase

Table 4.5. Determination of reciprocal viscosity values.

Incubation time (t) (min)	Time to flow (s)	Specific viscosity $n = (t - t_0)/t_0$	$1/n$
0	302.75	11.75	0.085
5	247.12	9.41	0.106
10	211.34	7.90	0.127
14	190.76	7.04	0.142
17	174.47	6.35	0.157
21	162.45	5.84	0.171

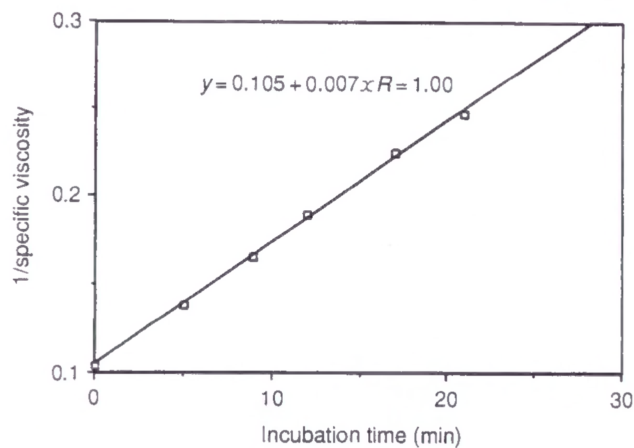

Fig. 4.11. Plot of reciprocal viscosity ($1/n$) against incubation time.

Extraction

1. Mill a feed sample (approximately 100 g) to pass a 0.5 mm screen and mix thoroughly.
2. Weigh samples of the above feed (0.5 ± 0.01 g in quadruplicate) into glass test tubes (16 × 120 mm).
3. Treat each sample with 5 ml of 0.1 M sodium acetate buffer (pH 4.7) and stir on a vortex mixer. To two of these tubes, add water (0.2 ml) with stirring, and to the other two tubes add control *Trichoderma* sp. xylanase (0.2 ml, 1360 mU) with vigorous and immediate stirring on a vortex mixer.
4. Leave the slurries at room temperature with occasional stirring on a vortex mixer over the following 20 min.
5. Centrifuge tubes (3000 rpm, 10 min) in a bench centrifuge and use the supernatant directly. Assays should be initiated within 30 min of obtaining these extracts to minimize loss of enzyme activity.

Assay

1. Accurately transfer 0.5 ml aliquots of supernatant solutions (in duplicate) to glass test tubes (16 × 100 mm) at room temperature.
2. Add 0.5 ml of Azo-WAX to each tube and stir the tube vigorously. Immediately place the tube in a water bath set at 50°C ± 0.1°C and incubate for 30 min.
3. After exactly 15 or 30 min (depending on the level of enzyme activity), add 2.5 ml of industrial methylated spirits (IMS) and stir the tube vigorously on a vortex mixer. Store the tube at room temperature for 5 min. This treatment terminates the reaction and precipitates non-depolymerized dyed substrate.
4. Centrifuge the tubes at 3000 rpm for 10 min and measure the absorbance of the supernatant solutions at 590 nm against a reaction blank.

The reaction blank is prepared by adding 2.5 ml of IMS to a mixture of 0.5 ml of azo-wheat arabinoxylan and 0.5 ml of 0.1 M sodium acetate buffer (pH 4.6). The slurry is stirred and stored at room temperature for 5 min before centrifugation (3000 rpm, 10 min). A single reaction blank is required for each feed sample.

Calculation of activity

The level of xylanase in the flour sample is calculated as follows.

Activity in feed sample (0.5 g) = *Added activity* × *SA* / (*TA* – *SA*)

where *Added activity* is the amount of xylanase added to the feed slurry at the time of assay (in the control xylanase solution; 0.2 ml) (e.g. 1360 mU); *SA* is the reaction absorbance obtained for extracts of the feed to which no control xylanase was added; *TA* is the total absorbance (i.e. the absorbance of extracts of the sample to which the control xylanase was added).

Example calculation

Sample	Absorbance (590 nm)/ 30 min incubation
1. Feed A	0.000
2. Feed A containing *Trichoderma* sp. xylanase (*SA*)	0.502
3. *SA* + 1360 mU xylanase (in the assay) (*TA*)	0.908

Activity in 0.5 g of feed A = *Added activity* × *SA* / (*TA* – *SA*)

where *SA* = absorbance of extract of sample A assayed by the standard format (e.g. Abs = 0.502); *TA* = the total absorbance, i.e. the absorbance of extracts of sample A to which the additional xylanase (0.2 ml; 1360 mU) was added (e.g. Abs = 0.908).

Thus:

Activity in the feed (U per 0.5 g) = (1360 / 1000) U × [0.502 / (0.908 – 0.502)]

= 1.682 U.

U g^{-1} (or kU ton^{-1}) = 1.682 × 2000 = 3363.

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