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Measurement of *endo*-1,4-β-D-Xylanase

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Abstract

Various procedures for the measurement of xylanase in fermentation broths, commercial enzyme mixtures, bread improver mixtures and feed samples are described. Problems associated with the routine use of reducing-sugar based methods are highlighted and the advantages and limitations of viscometric and dye-labelled substrate procedures for measurement of trace levels of activity in feed samples are discussed.

1. INTRODUCTION

In recent years there has been a growing interest in the use of xylanase (1,4-β-**D**-xylan xylanohydrolase; EC 3.2.1.8) enzymes in the paper pulp, food and feed industries. In pulp processing, there is a desire to replace chemical bleaching procedures with more "environmentally friendly" technologies. The potential for the use of "cellulase-free" xylanases to assist in lignin removal from pulp through hydrolysis of xylan in lignin-xylan complexes has been suggested [1]. Controlled xylanase treatment of pulp has been reported to enhance its beatability and binding ability and thus its paper-making properties [2,3]. Such enzymes may also find application in the retting of flax and hemp to produce higher grade plant fibres [4].

The major endosperm, cell-wall polysaccharide of wheat and rye grains is arabinoxylan. In wheat flour, this polysaccharide is present in both a soluble and an insoluble form. There is considerable evidence suggesting that insolubility may be due to covalent cross-linking through fenilic acid residues. Water soluble pentosans (arabinoxylans) form highly viscous solutions and it has been estimated [5] and subsequently demonstrated [6,7] that more than 20% of the water in wheat-flour dough is associated with pentosans. Treatment of wheat dough (at the optimum water absorption level) with excess levels of xylanase results in a rapid loss of dough strength and production of a wet, sticky dough mass [6]. However, the judicious use of xylanase in bread-improver mixtures may lead to improved bread properties such as increased loaf volume or more desirable crust colour.

The water requirement in the fractionation of starch and gluten from wheat-flour slurries is about two cubic metres per ton of flour. Concentration of the wash water is hampered by the high concentration of starch and especially arabinoxylan present. However, treatment with α-amylase and xylanase followed by heating to 85°C in a jet cooker and whirlpool separation of denatured protein, allowed concentration of the liquor to over 60% total solids in a steam cooker [8].

The use of multi-enzyme systems for wheat, rye, barley and soya based poultry and piglet diets is rapidly becoming an accepted technology for improving feed digestibility and feed-conversion ratios [9]. These mixtures contain xylanase and cellulase enzymes which reportedly depolymerise arabinoxylan and mixed-linkage beta-glucan, thus removing their viscosity building properties. This results in more rapid and more efficient absorption of nutrients from the gut. However, these enzymes are generally added to feeds pre-pelleting, and there is some question as to how much activity actually survives pelleting. The measurement of this activity is complicated by several factors including: (a) the low levels of activity present; (b) the presence of high concentrations of reducing compounds (which interfere with reducing-sugar based assays); (c) the presence of high concentrations of substrate in the feed (eg. wheat arabinoxylan); and, (d) the possibility of non-specific adsorption of the enzyme to insoluble fibre materials in the feed.

From the above examples it is clear that xylanase is finding widespread industrial application, and the scope for new applications is restricted mainly by the limited availability of specific xylanases with the required purity, properties (ie. pH optima, thermal stability) and action patterns [10]. To aid in these developments, simple, reliable and sensitive procedures are required for the specific and quantitative measurement of xylanase in a range of products ranging in enzyme activity from very high levels to trace quantities. In the remainder of this communication, procedures currently used for the measurement of xylanase will be discussed in terms of their advantages and limitations, and a new chromogenic substrate in tablet form (XylaZyme) will be introduced.

2. MEASUREMENTS OF XYLANASE BY REDUCING-SUGAR PROCEDURES

For the specific measurement of β-xylanase, the single major requirement is a pure polysaccharide substrate. Many of the commercially available xylan preparations are highly contaminated with starch and in some cases (oat xylan) with mixed-linkage β-glucan. The starch and β-glucan must be removed either by fractionation or by enzyme treatment (α-amylase; lichenase) followed by solvent precipitation and recovery, before they can be used with confidence.

2.1 Dinitrosalicylic Acid (DNS) procedure

Factors contributing to the non-linearity of enzyme assays which incorporate the detection of reaction products using dinitrosalicylic acid (DNS) have been discussed by Bailey [11]. He concluded that the two major factors which cause non-linearity are: (a) the practice of diluting reaction products before

quantification of reducing compounds; and (b) insufficiency of substrate. The DNS procedure which is most commonly employed, uses equal volumes (1.0 ml) of enzyme and substrate (10 mg/ml) with addition of 20 ml of water after boiling with DNS (2 ml), and is effective over the range of 1–5 mg xylobiose equivalents per assay. Thus, a high proportion of the substrate must be cleaved to give measurable absorbance values. Under these conditions, two other factors will come into play, namely product inhibition and transglycosylation. In this latter process, a glycosidic bond is cleaved, but, the oligosaccharide fragment (the glycosidic bond of which was cleaved) is transferred to an oligosaccharide reaction product rather than to water. This results in no nett increase in the level of reducing end groups. This reaction is commonly reported [12–14] in the hydrolysis of 1,4-β-**D**-polysaccharides, and becomes much more pronounced as the concentration of oligosaccharides in the reaction mixture increases ie. as the reaction proceeds.

The problem associated with insufficiency of substrate is likely to be much more pronounced if a highly branched arabinoxylan or 4-**O**-methylglucuronoxylan is used instead of a xylan with a low degree of branching such as beechwood xylan.

Another major problem with the DNS procedure is the non-stoichometry of the colour response with equimolar concentrations of oligosaccharides of increasing degree of polymerisation ie. 1 μmole of xylobiose gives a greater colour response than 1 μmole of xylose. This is thought to be related to the highly alkaline nature of the DNS reagent mixture. This problem could be minimised by using xylobiose instead of xylose as the standard. In the measurement of α-amylase action on starch using DNS, maltose is used as the standard; and in the case of cellulase action on modified celluloses or β-glucan, cellobiose is commonly employed.

2.2 Nelson/Somogyi procedure

The major advantages of this procedure [15] are: (1) the colour response with oligosaccharides of increasing degree of polymerisation is stoichiometric; and, (2) the assay is very sensitive (10–50 μg range). The high sensitivity means that a much smaller degree of hydrolysis of the substrate is required to yield measurable reaction colours; thus the enzyme will be acting at the most accessible regions in the substrate and the likelihood of product inhibition and/or transglycosylation is minimised. In our hands, the same initial rates of hydrolysis of birchwood xylan (uronic acid:xylose = 13:87) and highly substituted rye-flour arabinoxylan (Ara:Xyl = 49:51) were obtained with a crude *Aspergillus niger* xylanase preparation; similar results were obtained with a *Trichoderma reesei* preparation. In all cases, the reaction rate curve was linear over the absorbance (510 nm) range of 0.1–0.8.

The major disadvantage with the Nelson/Somogyi procedure is that when the copper reagent is added, the xylan substrate precipitates, and a turbidity is often observed in the final reaction tubes even after acidification. This turbidity is most pronounced in reaction blank solutions. Thus, it is necessary to centrifuge the final reaction solutions before the solution colours are measured.

2.3 General considerations

Reducing sugar methods can be used to measure enzyme activities in preparations which, after appropriate dilution, contain low levels of background reducing compounds. The methods can be used to measure enzyme activities in partially purified fermentation broths (ie. diafiltered) and commercial xylanase enzyme preparations. However, they cannot be applied to the measurement of activity in bread improve mixtures, cereal flours or animal feed mixtures to which enzymes have been added.

Reducing-sugar procedures are non-specific and a range of enzyme activities could potentially contribute to the final value. Thus, with birchwood xylan as substrate, other activities such as β-xylosidase and α-glucuronidase would be measured as well as *endo*-xylanase; with arabinoxylan as substrate, α-**L**-arabinofuranosidase would be measured along with *endo*-xylanase. However, in reality, the level of xylanase in commercial preparations of this enzyme are generally so much higher than the other activities, that the contribution of the latter to the final reducing sugar value is negligible.

3. VISCOSIMETRIC METHODS

The major advantages of viscosimetric methods are specificity and sensitivity [9,16]. *endo*-Cleavage of rye-flour arabinoxylan by highly purified *Trichoderma sp.* xylanase (Figure 1) results in a rapid viscosity decrease even at very low enzyme concentrations (0.01 Units). In contrast, *exo*-acting α-**L**-arabinofuranosidase gives negligible viscosity decrease over an incubation period in which the arabinose content of the polysaccharide is reduced form 49 to 43%. With polysaccharides, such as rye arabinoxylan, which give high solution viscosities, assays based on viscosity decrease are about ten times more sensitive than the Nelson/Somogyi reducing sugar procedure. Hydrolysis of as little as 0.1% of the glycosidic linkages in the 1,4-β-**D**-xylan backbone can be detected. Furthermore, since the assays are unaffected by even high concentrations of low molecular weight sugar compounds, they can be used to measure tract levels of enzyme activity in animal-feed materials.

The major disadvantage of viscometric procedures are that they are time consuming and only a limited number of assays can be run concurrently. Furthermore, the decrease in viscosity with increase in enzyme concentration is exponential, not linear. also, the rate of change in viscosity is highly dependent on the initial viscosity of the substrate solution.

4. CHROMOGENIC SUBSTRATES

Many of the problems experienced in the assay of *endo*-xylanase can be resolved by the use of chromogenic or dye-labelled xylan-type polysaccharides. Such substrates may be soluble [17,18] or rendered insoluble through covalent cross-links [19] and have as major advantages the fact that they allow the measurement of xylanase in materials containing high levels of reducing sugars

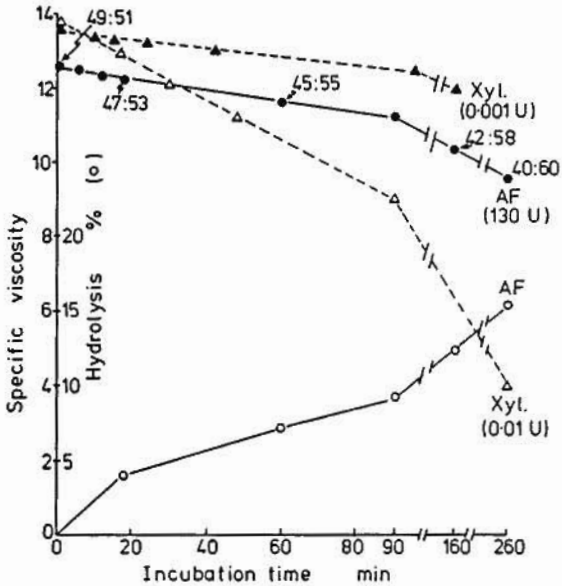

Figure 1. Hydrolysis of rye-flour arabinoxylan by highly purified *Trichoderma* sp. xylanase and *A. niger* α-**L**-arabinofuranosidase. Rye arabinoxylan (15 ml, 1% w/v) in 0.1 M sodium acetate buffer (pH 4.7) was treated with arabinofuranosidase (1 ml, 130 U on *p*-nitrophenyl α-**L**-arabinofuranoside) or xylanase (0.001 or 0.01 U on rye arabinoxylan) at 40°C. At various time intervals, viscosity measurements were taken and aliquots were removed for reducing-sugar determinations. Numbers (49:51) are the Ara:Xyl ratio of the polysaccharide. Xyl is xylanase treatment and AF is arabinofuranosidase treatment.

e.g. animal feed preparations and bread improver mixtures; they allow specific measurement of xylanase in the presence of high concentrations of *exo*-acting enzymes such as α-**L**-arabinofuranosidase; they form the basis of assays which are simple, quantitative and reproducible; they relate directly to assays based on viscosity reduction and thus more accurately reflect the likely significance of a given concentration of a particular type of enzyme ie. these assays can be used to directly compare xylanases from different sources in a particular industrial application; and, they can be used over a wide range of temperature and pH conditions.

The single major disadvantage with these substrates is that the chemistry of dyeing of the xylan substrate cannot be accurately controlled, thus each production batch of the substrate must be standardised with a particular enzyme before use.

The major requirements in the preparation and use of chromogenic substrates for the assay of *endo*-xylanase are: (1) the availability of highly purified, soluble polysaccharides of moderate viscosity; (2) physical, chemical and microbiological stability of the polysaccharide in solution over extended periods of storage; (3) a simple assay format and an effective procedure to quantitatively separate hydrolysed and non-hydrolysed dyed polysaccharide fragments; (4) adequate dyeing of the polysaccharide substrate to give linear, or near linear, release of dyed fragments over the absorbance range 0.1–1.0 absorbance units; and (5) a final dye-labelled substrate which is very susceptible to enzyme attack.

4.1 Soluble chromogenic xylan substrates

Soluble chromogenic substrates for the assay of xylanase have been prepared from oat-spelt xylan, beechwood and birchwood 4-**O**-methyl-**D**-glucurono-**D**-xylans and wheat-flour arabinoxylan. In general, these substrates are not totally soluble, but contain some colloidal dyed-xylan material as well. Wheat arabinoxylan dyed with Remazol Brilliant Blue R (Azo Wheat-Arabinoxylan) at a concentration of 1% w/v tends to settle from solution on prolonged storage at 4°C. However, complete redissolution is achieved by warming the substrate to 40°C followed by vigorous shaking of the container plus contents.

Assays employing soluble chromogenic xylan substrates involve incubation of an aliquot of soluble dyed substrate with enzyme solution under defined conditions of temperature and pH, with termination of the reaction and precipitation of non-depolymerised substrate by the addition of an organic solvent or an organic solvent/salt solution. Precipitated material is removed by centrifugation and the colour in the supernatant solution is measured. The colour is directly related to the enzyme concentration by reference to a standard curve. Typical curves relating the concentration of a *Trichoderma sp.* xylanase to absorbance increase on incubation with two dyed xylan compounds is shown in Figure 2.

4.2 Insoluble chromogenic xylan substrate

Such substrates are prepared from soluble polysaccharides, or dye-labelled, soluble polysaccharides and rendered insoluble by covalent crosslinking with agents such as epichlorohydrin. In these substrates, the polysaccharide molecules are "locked" into a three-dimensional conformation. The susceptibility of the substrate to xylanase attack is influenced by the concentration of the crosslinks; the size (length) of the crosslinking agent; the concentration of the dye molecules attached to the polysaccharide; and the degree of natural substitution of the native polysaccharide. Problems associated with dispensing a solid substrate can be alleviated by incorporation of the substrate into tablet form. The only commercially available tabletted xylanase substrate is XylaZyme from MegaZyme (Aust.) Pty Ltd. The substrate is a dyed and crosslinked, highly purified birchwood xylan.

Assays employing XylaZyme tablets involve the addition of the tablet to suitably diluted enzyme preparation (1 ml) in sodium acetate buffer at 40°C. The tablet is designed to totally disintegrate without agitation within 20 seconds. The reaction is terminated after 10 min by addition of 10 ml of an alkaline solution

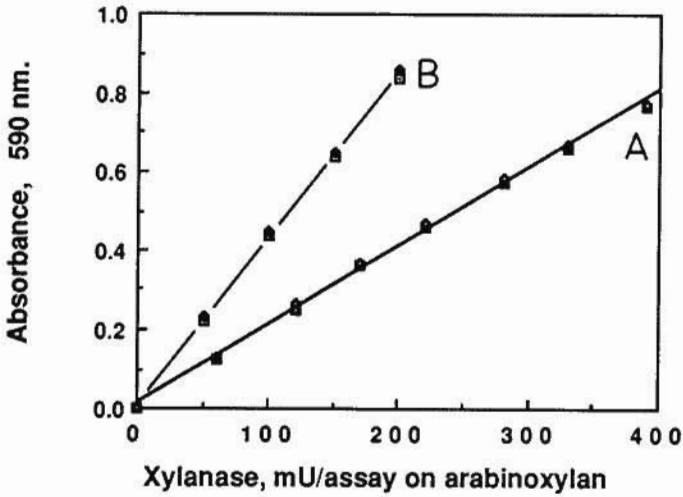

Figure 2. Release of dyed fragments soluble in aqueous ethanol from Azo-xylan (oat spelt, A), and Azo-wheat arabinoxylan (B) on treatment with pure *Trichoderma* sp. xylanase.

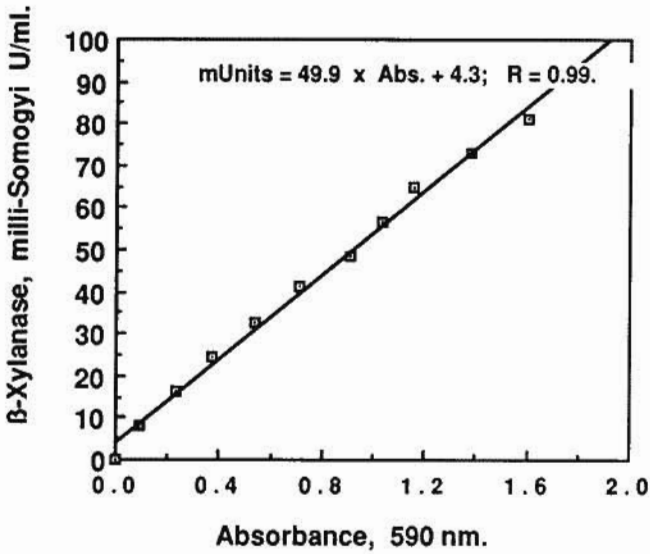

Figure 3. Standard curve relating *A. niger* β-xylanase enzyme Units to absorbance increase at 590 nm on hydrolysis of XylaZyme tablets. Standard assay conditions were employed and the reaction was terminated with 10 ml of Trizma base.

(Trizma base, Sigma Chemical Co.) with stirring; the slurry is filtered and the absorbance of the filtrate (at 590 nm) is measured. Enzyme activity is determined by reference to a standard curve (Figure 3).

The major advantages of these substrates over soluble chromogenic substrates are greater stability, sensitivity and ease of use. Assays based on the use of XylaZyme tablets are about 5-times more sensitive that assays employing soluble dyed wheat-flour arabinoxylan. This greater sensitivity allows the use of the substrate in the measurement of the low levels of xylanase present in chicken and pig feeds pre- and post-pelleting.

Xylanase and cellulase-type enzymes are added to wheat- and barley-based chicken and pig diets to depolymerise arabinoxylan and beta-glucan. This results in a decrease in viscosity of the gut contents and an improved absorption of nutrients, with resulting increases in feed conversion ratios. In studies of enzymic methods for use as predictors of *in vivo* response to enzyme supplementation of barley-based diets when fed to young chicks, Rotter *et al.* [9] found that the most reliable assay was based on the use of Azo-barley glucan (dyed barley β-glucan). Similar studies have not, as yet, been performed on xylanase assay procedures. However, in preliminary studies we have shown that

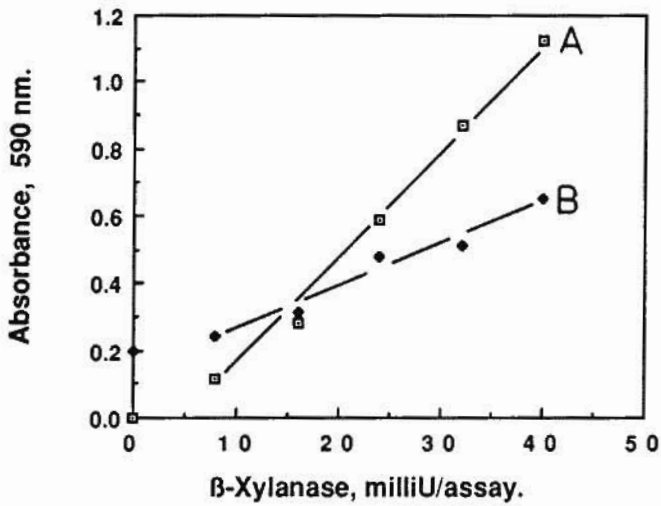

Figure 4. Effect of feed material on the assay of *Trichoderma* sp. xylanase using XylaZyme tablets. Enzyme solution (2.0 ml, 0-50 mU) in 25 mM sodium acetate buffer (pH 4.7) was incubated with stirring at 50°C in the presence (B) or absence (A) of feed material (0.2 g) for 10 min. the reaction was terminated after 10 min by the addition of 6 ml of 1% Trizma base. The solution was filtered and the absorbance at 590 nm measured. The absorbance of ~0.2 for the feed sample with no exogenous xylanase added in the assay procedure, was due to the presence of xylanase in this feed sample.

XylaZyme tablets have the required sensitivity. In the assay procedure employed, a milled feed sample (0.2 g) in buffer (2 ml) is incubated at 50°C with stirring. A XylaZyme tablet is added and the reaction is allowed to proceed for 30 min. The reaction is stopped by the addition of Trizma base (6 ml) and the slurry is filtered.

With this assay it has been possible to demonstrate some of the problems associated with the measurement of trace levels of enzyme activities in feed samples. In Figure 4, the relationship between xylanase concentration (*Trichoderma* sp.) and absorbance, in the presence (B) and absence (A) of added feed sample (0.2 g) is shown. It is evident that in the presence of the feed material, the slope of the standard curve is significantly reduced. This could be due either to non-specific binding of the enzyme to feed components or alternatively to the competitive action of alternative substrate material in the feed mixture. It is essential that the nature of this effect is identified and, if possible, that assay strategies to remove it be developed. The need for accurate and reliable assays for the measurement of xylanase, cellulase, β-glucanase and α-amylase in animal feeds pre- and post-pelleting is essential to allow the control of processing conditions and for regulatory purposes.

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