Use of Xylose Dehydrogenase from *Trichoderma viride* in an Enzymic Method for the Measurement of Pentosan in Barley

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ABSTRACT

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A xylose dehydrogenase has been isolated from *Trichoderma viride*. It has been used as part of an enzyme-based assay to measure pentosan in barley. Pentosan is extracted with perchloric acid and then hydrolyzed by a cocktail of enzymes including xylanase, arabinofuranosidase, feruloyl esterase and acetyl esterase. Some 92% conversion to xylose was obtained, and this is measured in turn using a xylose dehydrogenase-based assay mixture involving NAD-dependent reduction of phenazine methosulphate monitored from absorbance increase at 585 nm. The xylose dehydrogenase is somewhat unstable and this will need to be enhanced for the assay to be functional.

Key words: Assay, enzymes, pentosan, xylose, xylose dehydrogenase.

INTRODUCTION

Arabinoxylan is a substantial component of cell walls in many plant systems[8]. In the processing of those plant tissues, for example in the production of certain foodstuffs, these pentosans can have a number of impacts. For example, they influence dough formation in bread making[14] and may retard solid-liquid separation in brewing[18] as well as causing quality challenges, such as haze in beer[4]. High viscosity levels due to pentosans can impede the nutritive value of cereals for poultry[3].

One of the challenges in the study of arabinoxylans is their measurement. Whilst a colorimetric procedure is available[5], there are a number of shortcomings to it. Notably it involves the subtraction of absorbances at two wavelengths, which invariably leads to some exaggeration of error and furthermore the absorbance values obtained are very sensitive to the reaction time between the sample and the reagents (a mixture of acetic and hydrochloric acids, phloroglucinol and glucose).

Recently we have been using purified carbohydrases in studies of the architecture of the walls surrounding the cells of the starchy endosperm of barley[9,11]. Amongst these enzymes have been an acetyl esterase and feruloyl esterase, which cleave acetyl and feruloyl groups from the pentosan; an arabinofuranosidase, which splits arabinose side chains from the xylan backbone; and a xylanase, which digests the xylan backbone. We reason that if a xylose dehydrogenase were available, then using the battery of enzymes we could develop an assay procedure for arabinoxylan with quantification related to the extent of NAD reduction (Fig. 1). In this paper we report the isolation of a xylose dehydrogenase from *Trichoderma viride* and its use in a nascent version of such an assay.

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MATERIALS AND METHODS

Materials

All enzymes other than xylose dehydrogenase were a gift of Novozymes Biotech, Inc., Drew Avenue, Davis, CA. All other materials were from Fisher (www.fishersci.com) unless otherwise indicated.

Growth of *Trichoderma viride* and extraction of xylose dehydrogenase

T. viride NRRL 3652 was grown on a 2L scale for 3 days at 30°C as described in Kanauchi and Bamforth[10], using 1% (w/v) xylose as the sole source of carbon and energy. Mycelia were harvested by filtration (Fisher Sci-

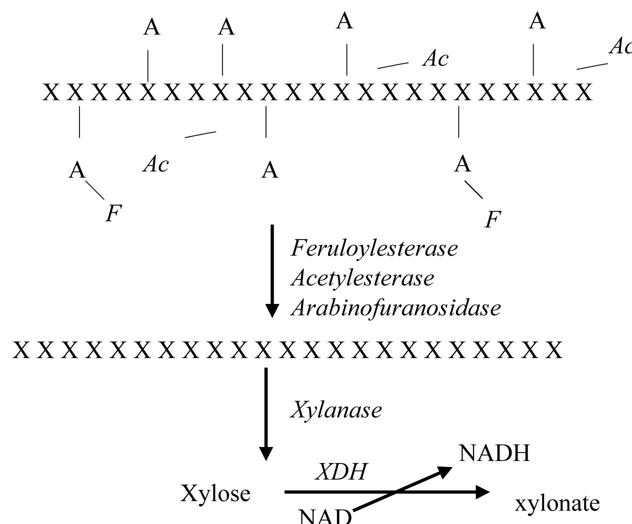

Fig. 1. The principle of the proposed assay for xylan: X = xylosyl; A = arabinosyl; F = Feruloyl; A = Acetoyl.

entific, G6 cat no. 09-804-70A) and washed using 0.9% (w/v) sodium chloride. To the mycelia was added 10 mL of 50 mM sodium phosphate buffer, pH 8.0 containing 2 mM EDTA and 2 mM 2-mercaptoethanol. The mycelia were crushed with 40 g glass beads with the addition of solid carbon dioxide. The "crude extract" was recovered by centrifuging at 1075 g for 30 min.

Partial purification of xylose dehydrogenase

The crude extract was taken successively to 30, 40, 50, 60, 70 and 80% saturation by the addition of solid ammonium sulfate at 4°C. Precipitates were recovered by centrifuging for 10 min at 11,950 g and the pellet fractions redissolved in the extraction buffer before dialyzing against the same buffer. The dialyzed sample of the recovered 40–50% saturation precipitate was applied to DEAE-agarose (300 × 15 mm; Whatman, cat. no 4053025) which was then washed with 50 mL of 50 mM sodium phosphate buffer, pH 7.0. Subsequent elution was with a linear gradient (0–500 mM) of sodium chloride in the same buffer, at a flow rate of 60 mL/h. Fractions containing significant levels of xylose dehydrogenase activity were pooled and dialyzed against the original extraction buffer.

Polyacrylamide gel electrophoresis

This was carried out according to Reisfeld et al[17]. A stacking gel of 4.75% polyacrylamide at pH 6.8 and a separating gel of 10% polyacrylamide at pH 8.8 were employed. Enzyme activity was detected on the gel by a procedure adapted from the alcohol dehydrogenase method of Manchenko[12]. Gels were steeped in a solution containing 100 mM Tris-HCl, pH 9.0, 40 mg NAD, 10 mg nitro blue tetrazolium, 1 mg phenazine methosulfate and either 50 mM xylose or glucose at 25°C for 30 min. After staining, gels were fixed in 50% methanol contained 0.5% acetic acid.

Protein

This was measured by the procedure of Bradford[2], using bovine serum albumin fraction V as standard.

Assay of xylose dehydrogenase

Assays contained 100 mM sodium carbonate buffer (pH 11), 1.0 mL; 0.3 M D-xylose, 0.05 mL; 50 mM NAD, 0.05 mL; and enzyme in a total assay volume of 1.2 mL. After 15 min at 25°C 1.0 mL of a solution comprising 1 mM tetrazolium chloride, 0.1 mM phenazine methosulfate and 1% Triton X-100 in 0.5 M sodium phosphate buffer pH 6.0 was added and the mixture left for 5 min prior to measurement of A_{585}. The method was calibrated using NADH standardized from the measurement of A_{340} (using a molar extinction coefficient of 6.22×10^3 L mol^{-1} cm^{-1}). One unit of xylose dehydrogenase is the amount of enzyme that produces 1 µmol NADH per minute under the assay conditions.

Determination of pH stability of xylose dehydrogenase

An aliquot (0.5 mL) of enzyme solution was added to 0.5 mL of various pH buffers: 100 mM citric acid (pH 4.0–6.0), 100 mM phosphate (pH 6.0–8.0), 100 mM boric (pH 8.0–9.0), sodium carbonate (pH 9.0–11.0), Lysine-NaOH (pH 11.0–12.0) and left at 4°C for 4 h, prior to assay.

Colorimetric assay for pentosan

This was as described by Douglas[5].

Enzymic assay of pentosan

Pentosan was extracted from barley using perchloric acid[1]. After neutralization with 50 mM NaOH, extracts (0.05 mL) were added to 1 mL 100 mM citrate buffer (pH 6.0) containing 65.3 units of xylanase, 8.2 units of arabinofuranosidase, 3.6 units of xyloacetylesterase and 0.6 units of feruloyl esterase. These were the same enzyme preparations described by Kanauchi and Bamforth[9]. Xylanase II was used. After 120 min at 40°C, 0.1 mL of the reaction solution was added to 1 mL 500 mM sodium carbonate buffer (pH 11) to which had been added 0.05 mL 50 mM NAD and 0.1 mL (13 units) of xylose dehydrogenase partially purified as described earlier. The mixture was held at 25°C for 30 min before assaying the NADH produced as described in the xylose dehydrogenase assay above.

RESULTS AND DISCUSSION

Xylose dehydrogenase from *Trichoderma viride*

Trichoderma viride growing on xylose as sole source of carbon and energy did not release xylose dehydrogenase (XDH) until more than 24 h had elapsed, by which time growth had started to occur. The activity of XDH was maximal after approximately 3 days of culture, by which time growth had essentially reached a plateau.

Ammonium sulfate precipitation of crude extracts of the organism led to XDH being deposited in the 30, 40 and 50% saturation "cuts", with the highest specific activity for the enzyme being found in the third of these fractions. Additional purification was effected using ion exchange chromatography, with the enzyme eluting in a broad band over the NaCl concentration range 0.1–0.25 M. The specific activity of the pooled fractions was 1.21 munits per mg protein and, in the context of the assay design, no further purification of the enzyme was deemed necessary.

XDH from *T. viride* displayed maximal activity at pH 11. Similar enzymes from other organisms have also been shown to have pH optima in the alkaline range (see for examples references 19 and 20). In the present instance, no activity was displayed under the assay conditions used at pH values of 9 and lower, which is of value in terms of designing a specific assay for pentosan, as most enzymes that might interfere tend not to display much activity at such high pH values. The enzyme was much more stable at alkaline as opposed to acid pH values: there was less than a 10% loss in activity over 4 h at 4°C at pH's between 7 and 12, whereas there was a 60% loss in activity in the same period at pH 4 and a 20% loss of activity at pH 6. The enzyme was inactivated by freezing and was relatively heat sensitive. Whilst it retained over 80% of its activity in 4 h at 40°C (pH 8), it was much less stable at higher temperatures. This sensitivity of the enzyme is a potential weakness of the proposed assay and will need to be overcome if the regular production of fresh enzyme is

to be avoided. As yet we have not explored the utility of any stabilizers to protect the enzyme.

Xylose dehydrogenase catalyzes the oxidation of glucose as well as xylose. We are dealing with a single enzyme – both activities co-purified and migrated with identical R_F (0.928) on polyacrylamide gel electrophoresis at pH 8.8. Migration was towards the anode (+), which indicates that the enzyme is substantially anionic. The enzyme displayed an apparent K_m for xylose of 0.8 M (NAD concentration was 2.1 mM). This is much higher than the values reported for the enzymes from *Arthrobacter* sp. (17.4 mM[19]) and pig liver (NADP-linked, 8.8 mM[20]). The K_m for glucose was lower, albeit still high, at 0.22 M, but the V_{max} value for xylose was 4-fold higher than for glucose. The K_m value for arabinose was 1.45 M. When assayed at a fixed concentration in the assay of 100 mM, arabinose gave less than one tenth the reaction rate observed with xylose. We have not explored this enzyme in any further detail: it might be argued that it is a glucose dehydrogenase with ability to catalyze the oxidation of xylose (cf. Pire et al.[15]). Other glucose dehydrogenases have high K_m values, for example that from duck is reported to have a K_m of 2.0 M[13]. Providing the enzymes used to degrade pentosan have no contaminating glucanase activity, the ability of the enzyme to oxidize glucose is deemed not to be a problem for the proposed assay.

Using XDH to assay xylose

XDH was first employed in the assay of xylose. Price and Burling[16] previously reported an enzymic assay for xylose using glucose dehydrogenase from *Bacillus megaterium,* which displayed a K_m for xylose of 7.5 M. The advantage of using enzymes such as that employed by Price and Burling[16], and also that used here, is precisely the fact that they have these very high K_m values. Under conditions where the substrate concentration (*viz.* xylose) is very much lower than the K_m, then pseudo-first order kinetics pertain and the rate of reaction in a short time frame is proportional to the amount of substrate present. If the substrate concentration is less than $0.05 \times K_m$, then the deviation from linearity in an assay is theoretically less than 3.3%[7]. Of course, any other factor which can affect initial reaction rate (e.g. pH, temperature, presence of inhibitors or activators) must be rigorously controlled.

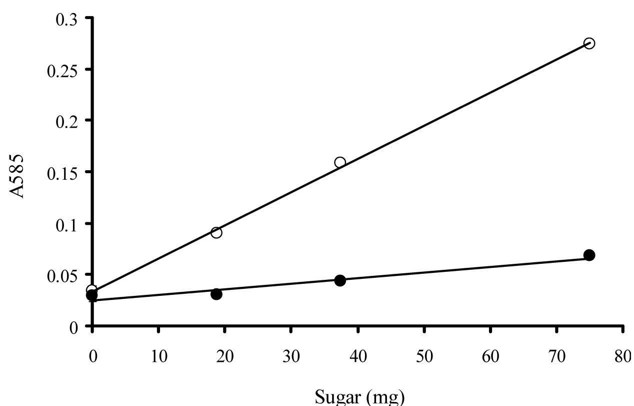

Fig. 2. The oxidation of xylose and glucose by xylose dehydrogenase. A fixed incubation period of 30 min was used: (○) xylose, (●) glucose.

In our assay, NADH was linked through to formazan formation as detected by absorbance at 585 nm[6] because, in later studies with cell wall derived material, interference with A_{340} was observed. As is illustrated in Fig. 2, however, there is a strong linear correlation between xylose concentration in the assay and A_{585} developed (in this case) after 30 min. A very low rate of color formation was observed in xylose-free samples. For the highest xylose concentration tested the absorbance developed is equivalent to approximately 0.5% oxidation of substrate, assuming a 1:1 stoichiometry between xylose and NAD. The much lower response for glucose in this time frame is apparent, reflecting the slower rate of conversion for this substrate. Reaction is not complete after 30 min. In any event it is apparent that, as this is a kinetic assay, any significant presence of glucose in the assay will lead to an inhibition of the rate of xylose oxidation. It is important that the contribution of any glucose is accounted for. In practice this means control assays in which the cocktail of xylanase, feruloyl esterase, arabinofuranosidase and acetyl esterase is omitted. As an (unexplored) alternative, test samples could be pre-treated with glucose oxidase to eliminate the interference.

Using XDH as part of an assay to measure xylan

Fig. 3 illustrates that xylose measurement in the assay after various periods of hydrolysis of xylan with xylanase was maximal at 2 h. Taking into consideration the water gained on hydrolysis it seems that the yield of xylose from xylan was approximately 92%, however xylose was the only soluble product detectable using thin layer chromatography. Maximum yields of xylose from pentosan extracted from barley depend on the presence of all four enzymes in the digestion cocktail (Fig. 4). There is clearly a sizeable hydrolysis of xylan in assays from which the xylanase was ostensibly absent. It seems that at least one of the other enzymes must contain a sizeable quantity of xylanase but this was not checked. It is also likely that there is some β-xylosidase present, as endo-β-1,4-xylan-

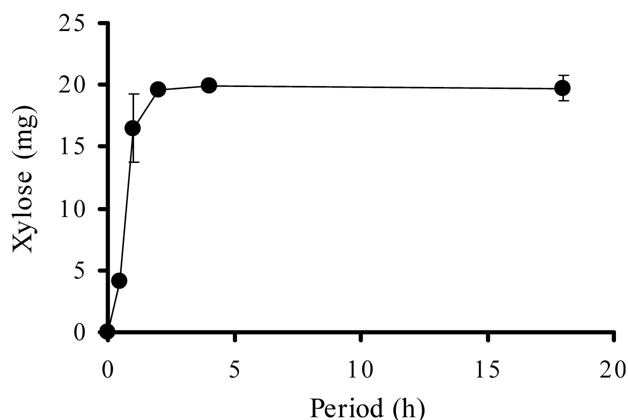

Fig. 3. Digestion of xylan by xylanase with assay of xylose via the xylose dehydrogenase reaction. Xylan (20 mg; Fisher cat # 22597-0250) was suspended in 1 mL of 100 mM citric Buffer, pH 6.0, containing xylanase (65.0 units). The mixtures was incubated at 40°C for 0–20 h and samples removed for assay of xylose in the xylose dehydrogenase assay described in the Experimental Section.

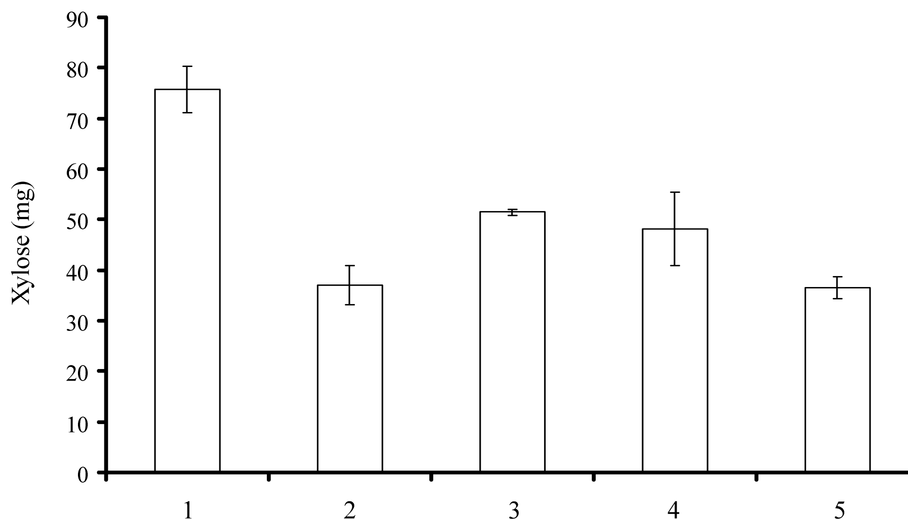

Fig. 4. The impact of omitting enzymes from the pentosan hydrolysis "cocktail" on the yield of xylose in the enzymic procedure for assessing pentosan. Sample 1 contained all the enzymes (xylanase, arabinofuranosidase, feruloylesterase, acetylesterase); Sample 2 lacked xylanase; Sample 3 lacked arabinofuranosidase, sample 4 lacked xyloacetylesterase; Sample 5 lacked feruloylesterase.

Table I. Pentosan measured by the present method and that of Douglas[5]. Samples examined were a crude cell wall preparation and a flour from dehusked barley prepared as described by Kanauchi and Bamforth[9].

	Pentosan (mg/g)	
	Enzyme method	**Douglas method**
Crude cell wall	69.22 (± 1.19)	63.13 (± 0.9)
Dehusked barley flour	19.83 (± 4.55)	15.22 (± 0.8)

ase will not convert xylan completely to xylose. Incubation of pentosan with xylose dehydrogenase as the sole enzyme caused no reduction of NAD (data not shown).

Table I shows the values for pentosan obtained using the present assay as compared to the procedure of Douglas[5]. Both methods were standardized using commercial xylan (Fisher cat # 22597-0250). The enzymic procedure gives somewhat higher values than does the colorimetric procedure. At this stage we have only investigated the method for the assessment of pentosan in barley. Pentosans from different sources are known to display different degrees of substitution with arabinose, and this could be expected to impact on both the digestibility of the polysaccharide with our cocktail of enzymes, and also the absolute value obtained. Assuming, however, that the degree of substitution of xylan with arabinose is relatively constant between samples of barley, then the present method retains its usefulness.

Further refinement of this method is necessary and, indeed, this might involve the identification of a xylose dehydrogenase with superior kinetic and stability properties to that reported here. Much more work is needed on establishing the general utility of the method for a diversity of cereal samples. However we believe that the basic principle of an enzyme-linked procedure for estimating pentosan levels in barley has been demonstrated. It would, of course, also be possible to use alternative procedures (e.g. high performance liquid chromatography) for assessing the hydrolysis products from the action of the initial enzyme cocktail. However the present procedure lends itself to those laboratories limited in their specialist equipment portfolio.

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