Novel and selective substrates for the assay of endo-arabinanase

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ABSTRACT

Substrates and assay procedures for the measurement of endo-1,5-α-L-arabinanase in crude, technical pectinase preparations have been developed. The method of choice employs carboxymethyl-debranched beet araban as substrate, and rate of hydrolysis is measured using the Nelson-Somogyi reducing-sugar procedure with arabinose as the standard. The substrate is physically and chemically stable in solution, and the assay procedure is simple, reliable and specific. Other assay procedures for the measurement of endo-arabinanase, which employ dyed debranched araban substrates, are also briefly described.

KEYWORDS

Debranched araban, endo-arabinanase, endo-galactanase, arabinofuranosidase, dyed debranched araban, fruit juice, arabinan haze.

INTRODUCTION

Commercial and consumer pressures on fruit processors to produce higher quality products at lower prices have necessitated the implementation of modern technology into traditional processes (Ducroo, 1987). In the processing of apples and pears, the yield of juice can be dramatically improved both by the use of enzymes to degraded pulp polysaccharides and by more exhaustive extraction of the pulp with diffusion equipment (Ducroo, 1987). Of course, these processes also significantly increase the amount of partially degraded polysaccharide which is solubilised. This polysaccharide material may be soluble as extracted, but subsequent changes in temperature and pH conditions can lead directly to precipitation (or crystallisation), or to chemical modification followed by precipitation. Such a problem has recently been experienced in the production of clear apple and pear juices, in which case, an arabinan haze material was identified. This material was shown to be microcrystalline linear 1,5-α-L-arabinan (Churms et al., 1983).

Arabinans, as present in cell-wall pectic-substances, have been shown to consist of a main chain of 1,5-α-linked L-

arabinofuranosyl residues to which other L-arabinofuranosyl residues are linked (1-3)-α and/or (1-2)-α in either a comb-like (Voragen _et al._, 1987) or ramified (Aspinall, 1984) arrangement.

It has been suggested (Whitaker, 1984) that the problem of "arabinan hazes" could be resolved by the use of pectolytic enzyme mixtures devoid of α-L-arabinofuranosidase. This enzyme produces 1,5-α-L-arabinans from highly branched, very soluble arabinans, by cleaving the 1,3-α and 1,2-α-linked L-arabinosyl branch units. However, since branched arabinans are not stable in juice and juice concentrates at certain steps of the manufacturing process, due to the low pH of the juice and high temperatures (at the heat treatment step), debranching can occur in the absence of arabinofuranosidase. Consequently, it is now generally accepted that the best solution to this problem lies in the use of pectinase enzyme preparations containing high levels of both arabinofuranosidase and _endo_-1,5-α-L-arabinanase. The arabinofuranosidase removes 1,3-α- and 1,2-α-linked arabinofuranosyl residues allowing ready access of the 1,5-α-L-arabinan main-chain to attack and depolymerisation by _endo_-arabinanase. The implementation of this technology is, however, limited by the absence of a simple and specific procedure for the measurement of _endo_-arabinanase in technical pectinase preparations. The substrate generally employed is linear 1,5-α-L-arabinan (Voragen _et al._, 1987) which is recovered by filtration of "hazy" fruit-juice concentrates. This substrate is costly to produce and thus difficult to obtain. Furthermore, assays employing this substrate (e.g. Hplc assays) are not specific. The substrate and its degradation products are cleaved by certain arabinofuranosidases, making the assignment of the relative contributions by each enzyme difficult.

The aim of the current research was to investigate the possibility of making a specific _endo_-arabinanase substrate from enzymically and chemically modified sugar-beet araban. Sugar beet araban is composed mainly of a 1,5-α-L-arabinan which is highly branched with 1,3-α- and 1,2-α-linked L-arabinofuranosyl residues. However, typical beet araban preparations also contain about 5-10% uronic acid and 8-15% D-galactose (Rombouts _et al._, 1988; McCleary, unpublished).

MATERIALS AND METHODS

α-L-Arabinofuranosidase, _endo_-1,5-α-L-arabinanase, _endo_ 1,4-β-D-galactanase and _endo_-polygalacturonanase were purified from a commercial pectinase preparation by ion-exchange and gel-filtration chromatographic procedures (McCleary, unpublished). Sugar-beet araban was purified from sugar-beet pulp according to the procedure of Jones and Tanaka (1965). This was further purified by application to a column of DEAE-Tris Acrylamide (pH 8.0) in 20 m_M_ Tris/HCl buffer (pH 8.0) (cf. Tagawa and Kaji, 1969). The araban fraction eluted unbound, and was precipitated by the addition of 5 volumes of ethanol. Alternatively, the pH of this araban solution (2% w/v araban) was adjusted to pH 4.5, and the solution treated for 4 h at 40°C with _endo_-1,4-β-D-galactanase and arabinofuranosidase to effect complete removal of

all the galactan regions which are susceptible to *endo*-galactanase and of the 1,3-α- and 1,2-α-L-arabinofuranosyl branch units. Debranched araban was recovered by alcohol precipitation, washed with ethanol, acetone and hexane and dried *in vacuo*.

Samples of this debranched araban were modified further as follows:

1. Carboxymethyl Debranched Araban. The debranched arabinan was treated with chloroacetic acid as previously described (McCleary, 1980) to give carboxymethylation degree of substitution values of approximately 0.05, 0.10 and 0.20. Following carboxymethylation, the CM-arabans were dissolved and treated with sodium borohydride (0.1 g/g) to remove the background reducing colour.
2. Red Linear Araban. Debranched araban was dyed with Procion Brilliant Red MX5B using a procedure similar to that described by Babson *et al*. (1970) for the dyeing of amylopectin.
3. Remazolbrilliant Blue- and Remazolbrilliant Black-CM-Linear Araban. CM-Debranched Araban was dyed with Remazolbrilliant Blue R or Remazolbrilliant Black B by the procedure previously described for the dyeing of barley beta-glucan (McCleary, 1986).

RESULTS AND DISCUSSION

A. Carboxymethyl Debranched Araban

In developing a substrate for the measurement of *endo*-arabinanase, it is essential that the activity values obtained relate directly to the activity of the enzyme on arabinan as found in fruit-juice concentrates. It is also essential that the assay specifically and reliably measures *endo*-arabinanase, even in crude enzyme mixtures. Furthermore, it is desirable that the assay is simple to use and that the substrate is easy to prepare and is chemically stable and gives solutions which are physically stable i.e. the polymer remains in solution.

An almost pure linear 1,5-α-L-arabinan fraction (Linear Arabinan) can be obtained by filtration of certain hazy fruit-juice concentrates. However, this process is very costly and greatly restricts the supply of this arabinan material.

In the current studies we have produced a material, termed "Debranched Araban" (it is not pure arabinan), from sugar-beet araban by a combination of chromatographic, enzymic and chemical procedures. A solution of beet araban is passed through a column of DEAE-Tris Acrylamide to remove most of the uronic acid containing material plus coloured material. The araban is then debranched with arabinofuranosidase and treated with *endo*-galactanase to remove sections (of galactan) which are susceptible to hydrolysis by this enzyme. The polysaccharide is recovered, alcohol washed and dried. It is then subjected to controlled carboxymethylation to improve solubility properties. Excessive carboxymethylation must be avoided as this reduces the susceptibility of the substrate to cleavage by *endo*-arabinanase.

The action of *endo*-arabinanase in a crude technical preparation containing 27 mU *endo*-arabinanase/0.2 ml (i.e. per assay) and

20 mU arabinofuranosidase/0.2 ml on these substrates, is shown in Fig. 1. Carboxymethyl (CM) Debranched beet Araban with a degree of carboxymethylation of 0.1, is hydrolysed at approximately 90% the rate for Linear Arabinan recovered from fruit juice concentrates. Increasing the degree of substitution (DS) with carboxymethyl groups to 0.2 seriously affects the susceptibility of the substrate to hydrolysis by endo-arabinanase. In fact, the rate is reduced to 59% the rate for Linear Arabinan. Decreasing the degree of substitution to DS 0.05 renders the substrate slightly more susceptible to cleavage by endo-arabinanase, but this substrate is more difficult to dissolve (similar to Linear Arabinan), and like Linear Arabinan, it precipitates from solution on short term storage at 4°C. Thus,

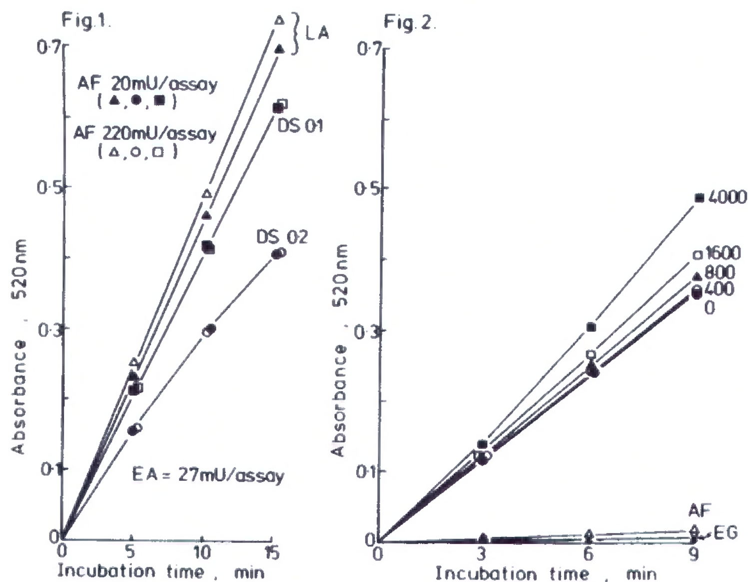

Fig. 1. Assay for endo-arabinanase (27 mU/assay) in a crude pectinase preparation using Linear Arabinan (LA) and Carboxymethyl Debranched beet Araban (DS 0.1 or 0.2) as substrate, in the presence of significantly different levels of arabinofuranosidase (20 or 220 mU/assay).

Fig. 2. Effect of arabinofuranosidase (0-4000 mU/assay) on the specificity of CM-Debranched Araban (DS 0.1) for the assay of endo-arabinanase (23 mU/assay). The effect of purified arabinofuranosidase (AF, 1000 mU/assay) and endo-galactanase (EG, 1400 mU/assay), in the absence of endo-arabinanase, on this substrate, is also shown.

the optimal degree of carboxymethylation is 0.1, as the substrate is easy to dissolve, remains in solution on dissolution, and is hydrolysed by endo-arabinanase at a rate very similar to that for the substrate of interest, namely Linear Arabinan from fruit-juice concentrates.

From Fig. 1 it is also evident that addition of purified arabinofuranosidase, at a level 10-times that in the crude preparation, has no effect on the absorbance values obtained for endo-arabinanase on the CM-Debranched Araban substrates. This clearly demonstrates the specificity of the assay procedure and substrate for the measurement of endo-arabinanase in the presence of arabinofuranosidase. [This arabinofuranosidase is type B described by Rombouts et al. (1988); it hydrolyses sugar-beet araban at about 50% the rate of para-nitrophenyl α-L-arabinofuranoside.] It is interesting to note that added arabinofuranosidase does have some effect when Linear Arabinan is the substrate.

The linearity of the assay procedure for highly purified endo-arabinanase (23 mU/assay) with time, on CM-Debranched Araban (DS 0.1), is shown in Figure 2. Also shown, is the action of arabinofuranosidase (AF, 1000 mU/assay) and endo-galactanase (EG, 1400 mU/assay) on this substrate, and the effect of added arabinofuranosidase (400-4,000 mU/assay) on the rate of hydrolysis by endo-arabinanase (23 mU/assay). It is evident that the substrate is resistant to attack by both endo-galactanase and arabinofuranosidase, and that contamination of endo-arabinanase with up to a 20-40-fold excess of arabinofuranosidase (400-800 mU/assay) has little effect on the specificity of the assay for endo-arabinanase. Consequently, the effect of arabinofuranosidase on the specificity of the endo-arabinanase assay procedure is of no practical significance. In a survey of a wide range of commercial pectinase preparations, the ratio of arabinofuraosidase to endo-arabinanase was, at maximum, 7.6:1.

The slight absorbance increase on treatment of CM-Debranched Araban (DS 0.1) with high levels of endo-galactanase are considered to be due to a very slight contamination of the endo-galactanase employed with endo-arabinanase (approximately (0.05%), rather than to a susceptibility of the substrate to endo-galactanase. A very similar absorbance increase was obtained on treatment of fruit-juice Linear Arabinan (which by g.l.c. is devoid of galactose) with this endo-galactanase preparation.

The major reaction products on cleavage of Linear Arabinan and Debranched Araban by endo-arabinanase are arabinobiose and arabinotriose, not arabinose. Since these oligosaccharides are not commercially available, it is convenient to use arabinose as the sugar standard in the reducing sugar assays. However, for this to be analytically valid, arabinose, arabinobiose and arabinotriose must give similar molar absorbance values with the reducing sugar method employed. The results shown in Figure 3 demonstrate that with the Nelson/Somogyi reducing sugar assay (Somogyi, 1952), this is the case.

The linearity of the assay procedure with concentration of endo-arabinanase is shown in Figure 4. The reaction curve is linear up to an absorbance of 0.8 i.e. with concentrations of purified endo-arabinanase up to 54 mU/assay under standard assay conditions (10 min, pH 4.5, 40°C).

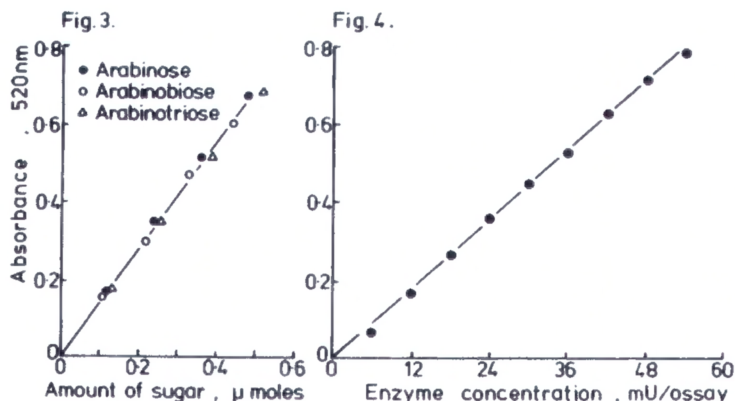

Fig. 3. Molar colour responses for arabinose, arabinobiose and arabinotriose with the Nelson-Somogyi reducing-sugar assay.

Fig. 4. Standard curve relating endo-arabinanase concentration to Nelson-Somogyi colour response under standard assay conditions (pH 4.5, 10 min, 40°C) with CM-Debranched Araban (DS 0.1).

The effect of substrate concentration on the rate of reaction is shown in Figure 5. The concentration of CM-Debranched Araban (DS 0.1) for half maximal velocity is 0.37 mg/mL. Thus, a concentration of 2.0 mg/mL in the reaction mixture is adequate, and is routinely employed.

These studies demonstrate that the substrate termed CM-Debranched Araban (DS 0.1) is very suitable for the assay of endo-arabinanase in crude enzyme mixtures. The substrate is readily solubilised and remains in solution on extended storage at 4°C, and under these conditions it is chemically stable for several months. (Addition of 2 drops of toluene to the substrate prevents microbial infection.) The substrate is hydrolysed by endo-arabinanase at a rate very similar to that for Linear Arabinan (from fruit-juice concentrates), and it is resistant to cleavage by arabinofuranosidase and endo-galactanase. Also, endo-polygalacturonanase has no action on the substrate (the substrate contains a small percentage of galacturonic acid). One limitation of this assay procedure is that it can't be directly used to

measure activity in enzyme preparations which contain a high level of reducing compounds (e.g. sugars; used as bulking agents in powder samples). With such samples, the reducing sugars must be removed before the assay is performed, or alternatively, a dyed-debranched araban substrate can be employed.

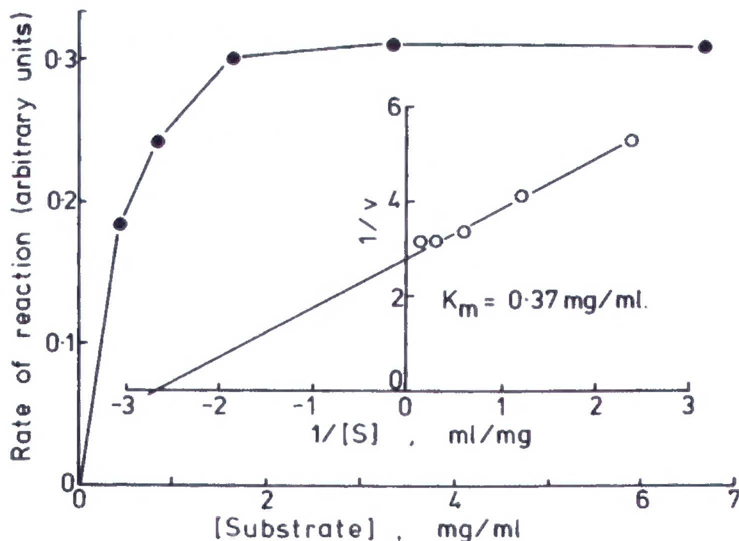

Fig. 5. Effect of substrate concentration on the rate of hydrolysis of CM-Debranched Araban (DS 0.1) by endo-arabinanase (purified from Gist Brocades C80 preparation).

B Dyed Debranched Araban

In the current studies, a range of dyed substrates have been prepared and these include Red Debranched Araban, (prepared with Procion Brilliant Red MX5B), Black CM-Debranched Araban (prepared with Remazolbrilliant Black B) and Blue CM-Debranched Araban (prepared with Remazolbrilliant Blue R). Each of these substrates is effective in the specific measurement of endo-arabinanase even in crude enzyme preparations containing high levels of reducing sugars. However, each suffers from certain limitations. With the Red Araban, the standard curve is sigmoidal, and it is very hard to produce substrates which give low background (blank) absorbances. The latter is not a problem with the Black and the Blue CM-Debranched Arabans. However, with these substrates it is difficult to get linearity over an acceptable absorbance range (i.e. about one Absorbance Unit). Work on these dyed substrates is continuing.

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