Measurement of Polysaccharide Degrading Enzymes Using Chromogenic and Colorimetric Substrates

Barry V. McCleary

MegaZyme (Australia) Pty Ltd, 6 Altona Place, North Rocks, NSW 2151

Enzymic degradation of carbohydrates is of major significance in the industrial processing of cereals and fruits. In the production of beer, barley is germinated under well defined conditions (malting) to induce maximum enzyme synthesis with minimum respiration of reserve carbohydrates. The grains are dried and then extracted with water under controlled conditions. The amylolytic enzymes synthesized during malting, as well as those present in the original barley, convert the starch reserves to fermentable sugars. Other enzymes act on the cell wall polysaccharides, mixed-linkage β-glucan and arabinoxylan, reducing the viscosity and thus aiding filtration, and reducing the possibility of subsequent precipitation of polymeric material. In baking, β-amylase and α-amylase give controlled degradation of starch to fermentable sugars so as to sustain yeast growth and gas production. Excess quantities of α-amylase in the flour result in excessive degradation of starch during baking which in turn gives a sticky crumb texture and subsequent problems with bread slicing. Juice yield from fruit pulp is significantly improved if cell-wall degrading enzymes are used to destroy the three-dimensional structure and water binding capacity of the pectic polysaccharide components of the cell walls. Problems of routine and reliable assay of carbohydrate degrading enzymes in the presence of high levels of sugar compounds are experienced with such industrial processes.

Enzyme activities, present in cereal and fruit products, or added during processing, are ideally assayed using the natural substrate and under conditions which simulate processing conditions. Polysaccharide *endo*-hydrolases aid processing by catalysing a viscosity reduction, and the ideal assay format would follow the reduction in viscosity with a natural substrate.[1] However, viscosity measurements are tedious and require considerable skill. Another routine assay for polysacharride degrading enzymes is the measurement of the increase in reducing sugar levels as the substrate is hydrolysed by the appropriate enzyme. The methods to estimate reducing-sugars include dinitrosalicylic acid (DNSA),[2] Nelson-Somogyi,[3] *p*-hydroxybenzoic acid hydrazide (PAHBAH) and ferricyanide procedures. However, with cereal and fruit products these procedures cannot be used because of the very high levels of reducing sugars present.

To overcome these problems, assays which exploit a specific reaction characteristic or a solubility property of a particular polysaccharide, have been developed. For example, the reaction of starch with iodine (to give a purple colour) and the decrease in this colour as the starch is depolymerised by α-amylase, is commonly employed to measure the activity of this enzyme.[4] The reaction of mixed-linkage β-glucan with Congo Red stain and Calcofluor, have been used to assay mixed-linkage β-glucanase activity in malt (malt β-glucanase).

Chromogenic Substrates

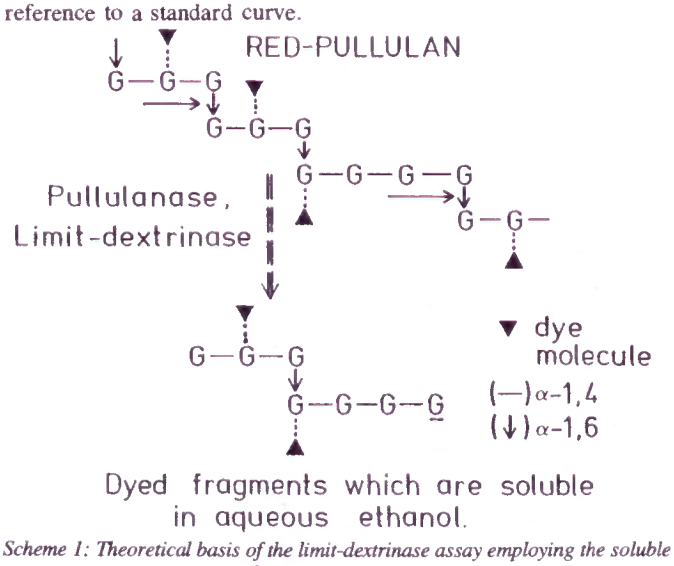
Many of the problems experienced in the assay of polysaccharides *endo*-hydrolases can be resolved by the use of chromogenic or dye-labelled substrates. Such substrates may be soluble[5,6] or rendered insoluble[7] through covalent cross-links, and have the following major advantages.

1. They allow measurement of enzyme activity in extracts containing high levels of reducing sugars e.g. the measurement of α-amylase, limit-dextrinase and malt β-glucanase in malted barley extracts.
2. They allow specific measurement of polysaccharide *endo*-hydrolases in the presence of high concentrations of *exo*-acting enzymes, e.g. the measurement of α-amylase in the presence of β-amylase in cereal flours, or the measurement of α-amylase in the presence of amyloglucosidase in industrial microbial enzyme preparations.
3. They form the basis of assays which are simple, quantitative and reproducible. In an RACI evaluation of azo-barley glucan for the assay of malt β-glucanase, which involved 18 laboratories and five malt samples, interlaboratory coefficient of variation values of less than 6% for each of the samples was obtained.
4. 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. This means that these assays can be used to directly compare the activity of the same enzyme from different sources (i.e. different microbial sources) in a particular industrial application. One such example of this is a comparison of the different β-glucanase enzymes which are used in the brewing industry to destroy the viscosity of β-glucan. Bacterial and fungal β-glucanases are employed, but for a given activity based on a reducing-sugar assay, bacterial enzymes are far more effective in viscosity reduction than are the fungal enzymes and this is reflected in assays based on the use of soluble dye-labelled barley β-glucan (azo-barley glucan).[8] Similar observations have been made in the evaluation of β-glucanases as supplements in chicken-feed diets[9] containing high levels of barley (and thus of β-glucan).
5. They can be used in assays over a wide range of temperature and pH conditions.

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

(a) Soluble chromogenic substrates

Soluble chromogenic substrates are produced by the controlled dyeing of highly purified soluble polysaccharides. Dyed polysaccharide is usually separated from unreacted dye by precipitation or by gel permeation chromatography. Assay formats employing these substrates generally involve the incubation of an aliquot of soluble dyed substrate with enzyme solution under defined conditions of pH and temperature, with termination of the reaction and precipitation of non-depolymerised substrate by addition of an organic solvent or an organic solvent/salt solution (Scheme 1). Precipitated material is removed by centrifugation and the colour in the supernatant solution measured. This colour can be directly related to the amount of enzyme in the assay mixture by reference to a standard curve.



Scheme 1: Theoretical basis of the limit-dextrinase assay employing the soluble substrate Red-Pullulan.

The major requirements in the production and use of soluble chromogenic substrates are:

(i) availability of highly purified, soluble polysaccharides of moderate viscosity (i.e. a viscosity which allows preparation of solutions of at least 1% w/v of polysaccharide in water),

(ii) physical, chemical and microbiological stability of the polysaccharide in solution over extended periods of storage,

(iii) a simple assay format and an effective procedure to quantitatively separate hydrolysed and non-hydrolysed dyed polysaccharide fragments,

(iv) adequate dyeing of polysaccharide substrate to give linear, or near linear, release of dye-labelled fragments over the absorbance range 0.1-1.0 absorbance units, and

(v) a final dye-labelled polysaccharide substrate which is very susceptible to enzyme attack.

(b) Insoluble chromogenic substrates

The substrates referred to here as insoluble chromogenic substrates are those which have been produced from soluble polysaccharides or dye-labelled, soluble polysaccharides and rendered insoluble by covalent crosslinking with a crosslinking agent such as epichlorohydrin. These substrates are far superior to, and should not be confused with, substrates which are prepared by dyeing insoluble polysaccharides, such as cellulose. These latter substrates are very resistant to enzyme attack and have little analytical use. With covalently crosslinked substrates, individual polysaccharide molecules are "locked" into a three dimensional conformation as a result of the crosslinking. The susceptibility of such substrates to enzyme attack is influenced by the concentration of the crosslinks, the size (or 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. The major requirements for useful and effective insoluble (covalently crosslinked), dye-labelled substrates are very similar to those outlined for soluble dye-labelled substrates.

Insoluble substrates have the inherent disadvantage that they must be weighed accurately into each assay tube, whereas soluble substrates are readily and accurately dispensed with commercially available liquid handling equipment such as the Eppendorf Multipette.® The problem of dispensing the insoluble substrate can, however, be overcome by providing the substrate in a tablet form. Such a substrate is available commercially for the assay of α-amylase (Phadebas tablets)[7] and for the assay of a range of polysaccharide *endo*-hydrolases, including α-amylase, limit-dextrinase, xylanase and *endo*-arabinanase, from MegaZyme (Australia) Pty Ltd.[10]

In the preparation of such tablets there are several essential requirements, namely: (i) the tablet must be sufficiently rigid to cope with normal handling and shipping requirements; (ii) on addition to buffer or buffered enzyme solution, it must disintegrate rapidly (i.e. within 20 seconds) and preferably without mechanical agitation (which leaves a varying percentage of the substrate attached to the walls of the test tube); and finally, bulking agents used in conjunction with the active component, must not interfere in the assay i.e. by acting as alternative substrates.

With novel, locally produced tablet substrates, the basic assay format involves the addition of the tablet to an aliquot (1 ml) of pre-equilibrated and correctly buffered enzyme solution. The tablet is designed to completely disintegrate without agitation within 20 s. The reaction is terminated after 10 min. by addition of 5 ml of an alkaline solution (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.

The major advantage of dyed, crosslinked substrates in tablet form is that they are stable indefinitely, and, unlike liquid substrates, there is little chance of back contamination of the bulk substrate preparation. Another advantage of these substrates is that, if prepared correctly and used in a good assay format, they are considerably more sensitive than their soluble counterparts. All reactions with the insoluble substrates are performed in aqueous solutions, whereas with soluble substrates, the reaction is generally terminated by the addition of an organic solvent to precipitate unhydrolysed material, and this format gives a different molecular size cut-off and a diminished sensitivity. For example, in the measurement of β-xylanase, assays based on the use of XylaZyme tablets, containing crosslinked and dyed birchwood xylan, are about 10 times more sensitive than assays employing soluble dyed arabinoxylan from wheat or soluble dyed oat-spelt xylan.

Barry V. McCleary is Technical Director of MegaZyme (Australia) Pty Ltd, a privately owned biotechnology company formed in 1989. Prior to this Dr McCleary was Principal Research Scientist with NSW Agriculture & Fisheries. He has been the recipient of Fulbright and Nuffield Foundation Scholarships, and in 1989 he was awarded the degree of Doctor of Science in Agriculture by the University of Sydney for his work on plant polysaccharides.

Colorimetric Substrates

Colorimetric substrates are based on the use of a defined oligosaccharide which is covalently linked to *p*-nitrophenol through the reducing D-glucosyl residue of the oligosaccharide. At present, such substrates are available only for the assay of α-amylase, β-amylase and amyloglucosidase. The action of these enzymes and of limit-dextrinase on starch is shown in Figure 1.

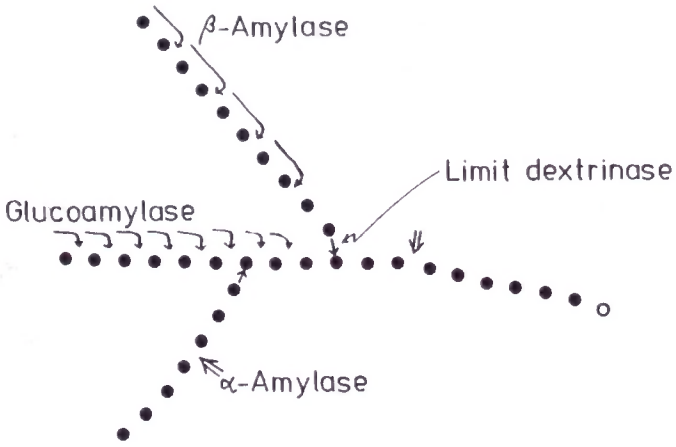
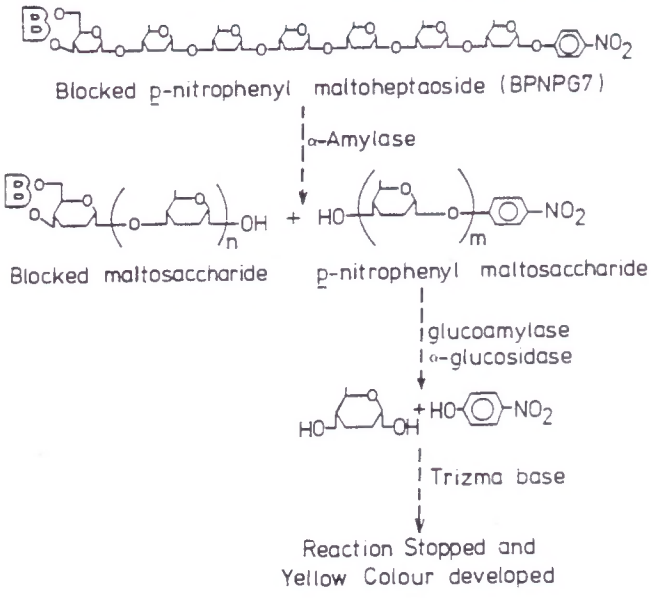


Figure 1: The action of starch degrading enzymes on amylopectin. ● ●, α-1,4-linked D-glucosyl residues; ● — ●, α-1,6-linked D-glucosyl residue; O, terminal, reducing D-glucosyl residue.

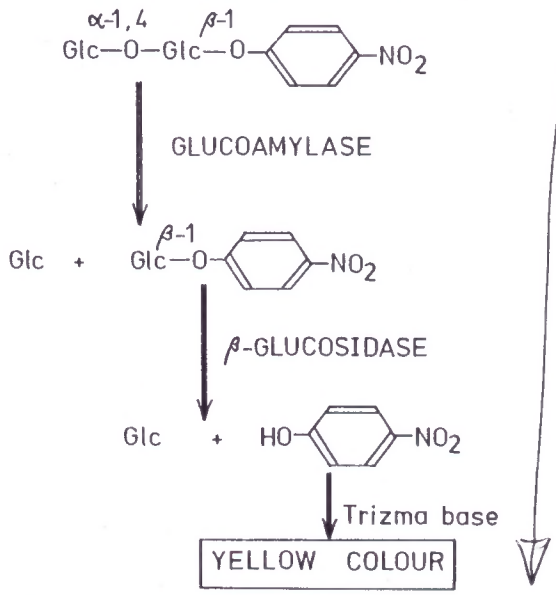
For the measurement of α-amylase, the substrate employed is "non-reducing-end blocked *p*-nitrophenyl maltoheptaoside" (BPNPG7).[11] This substrate is used in conjunction with excess quantities of amyloglucosidase and α-glucosidase, (which have no action on the native substrate due to the presence of the "blocking group"). On hydrolysis of the oligosaccharide by *endo*-acting-α-amylase, the excess quantities of amyloglucosidase and α-glucosidase which are present give essentially instantaneous and quantitative hydrolysis of the *p*-nitrophenyl maltosaccharide fragment to glucose and free *p*-nitrophenol. This assay format is shown in Scheme 2. A further advantage of this type of substrate is that, with minor modifications, it can be used to assay for different forms of the same enzyme in a mixture. A particular application of this technology is the ability to measure relative proportions of fungal and cereal α-amylases in bread improver mixtures. Cereal α-amylase rapidly hydrolyses blocked *p*-nitrophenyl maltoheptaose, but hydrolysis of the pentasaccharide substrate (blocked *p*-nitrophenyl maltopentaose) is slow. In contrast, fungal α-amylase hydrolyses both substrates rapidly, as a consequence of its different sub-site binding requirement at the active site of the enzyme. Fungal α-amylase is added to bread making flours to assist the hydrolysis of gelatinised starch to fermentable sugars during the bread proofing stage which assists in improving loaf volume and keeping quality. The enzyme is inactivated at temperatures above 50 °C, so it is effectively inactivated during the bread baking process and thus cannot cause excessive degradation of starch at this stage of the process (which would result in the production of a sticky crumb, and subsequent bread-



Scheme 2: Theoretical basis of the Ceralpha α-amylase assay procedure.

handling problems). Cereal α-amylases, being more thermostable, would have deleterious effects on bread quality if present in excess quantities in dough.

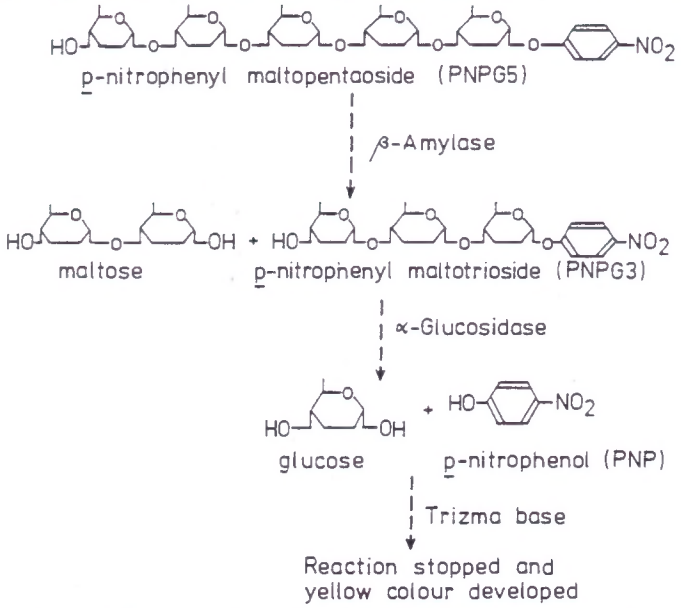
β-Amylase is an *exo*-acting enzyme which sequentially releases maltose units from the non-reducing terminus of amylose and amylopectin molecules. This enzyme can be effectively assayed in cereal extracts using *p*-nitrophenyl maltopentaose in the presence of yeast α-glucosidase (Scheme 3).[12] This assay takes advantage of the fact that cereal α-amylases have very limited action on maltopentaose (oligosaccharides containing at least seven 1,4-α-linked D-glucosyl residues are required for rapid hydrolysis). The assay also takes advantage of



Scheme 3: Theoretical basis of the Betamyl β-amylase assay procedure.

the fact that yeast α-glucosidase rapidly hydrolyses maltosaccharides having two or three D-glucosyl residues, but acts extremely slowly on maltopentaose. Thus, when β-amylase removes the terminal maltosyl unit from *p*-nitrophenyl maltopentaose, the resultant *p*-nitrophenyl maltotriose is rapidly hydrolysed by the yeast α-glucosidase, releasing free *p*-nitrophenol.

Another *exo*-enzyme active on starch fractions is the fungal enzyme amyloglucosidase which finds widespread industrial application. This enzyme is usually assayed with maltose as substrate, rather than starch, because the latter substrate is also rapidly hydrolysed by fungal α-amylase (which occurs at significant but varying levels in amyloglucosidase preparations). As an alternative to maltose, we have employed the substrate *p*-nitrophenyl β-maltoside in the presence of excess quantities of β-glucosidase (Scheme 4).[13] When the terminal α-linked



Scheme 4: Theoretical basis of the amyloglucosidase (glucoamylase) assay procedure.

D-glucosyl residue is removed by amyloglucosidase, the resultant *p*-nitrophenyl β-glucoside is instantaneously cleaved to glucose and free *p*-nitrophenol by the β-glucosidase. This assay format allows the measurement of amyloglucosidase in 10 min. instead of 60 min. required with conventional assay procedures.

Some Applications of Chromogenic and Colorimetric Substrates

(a) Measurement of α-amylase

Because of its industrial significance in the brewing and baking industries, numerous methods have been developed for the assay of α-amylase. Insoluble, dyed, cross-linked, starch-based, substrates in the form of tablets, form the basis of recommended procedures of the RACI Cereal Division, the American Association of Cereal Chemists (AACC) and the European Brewing Convention (EBC). Until recently, only one commercial tablet substrate was available for the assay of α-amylase.[7] These tablets have been very useful but are limited as they were developed for clinical applications and are thus buffered at pH 6.9, which is not the optimal pH for activity of fungal, cereal or bacterial α-amylases. Now, α-amylase assay tablets which are unbuffered[10] have become available, allowing greater flexibility of use. Materials for analysis are extracted and/or diluted in a buffer with the appropriate pH and ionic strength.

α-Amylase assay procedures employing the colorimetric substrate end-blocked *p*-nitrophenyl maltoheptaose[11] have many advantages over most other α-amylase assay formats. The substrate is available commercially in a ready-to-use form as Ceralpha α-amylase assay reagent. The assay simply involves the incubation of an aliquot (0.2 ml) of enzyme preparation with 0.2 ml of the substrate mixture for 10 min. at 40 °C, termination of the reaction and colour formation by the addition of an alkaline solution (1% w/v Trizma Base), and measurement of absorbance at 410 nm. In the clinical diagnostics field, this assay format is rapidly replacing most other procedures. However, in some laboratory situations (e.g. in flour mills, in industrial enzyme production facilities and in factories involved in enzyme blending) problems of contamination of the stock substrate solution with trace quantities of α-amylase (possibly from flour dust or aerosols) may be experienced.

(b) Measurement of β-xylanase

Xylanase enzyme is now finding widespread application in bread improver mixtures and in the enzymic bleaching of wood pulp. Activity is usually measured with the DNS reducing sugar assay[3] with a purified xylan from oat-spelts, larchwood or birchwood as substrate. These assays have various limitations, including the fact that reaction curves are not linear and that assays cannot be performed on materials containing high levels of reducing sugars. Alternative assay procedures involve the use of azo-xylan and azo-arabinoxylan and insoluble (covalently-crosslinked) dyed birchwood xylan in tablet form. This latter substrate is most widely applicable and is about 10 times sensitive

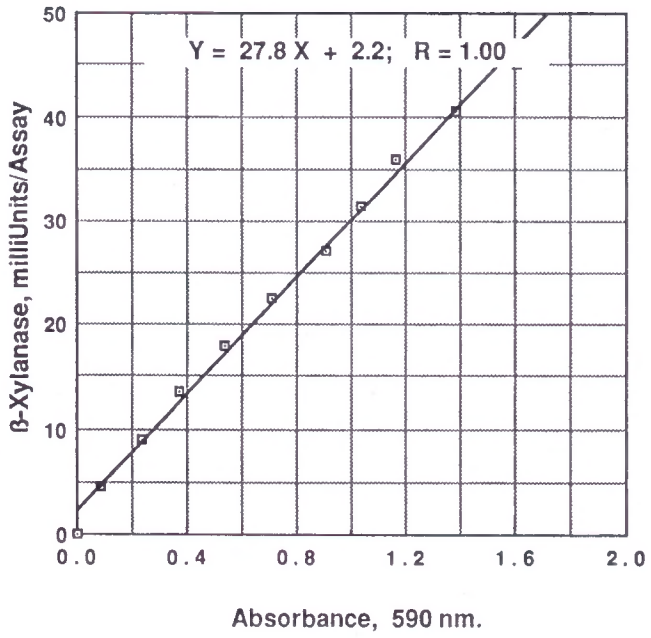


Figure 2: Standard curve for the action of xylanase from Aspergillus niger *on XylaZyme tablets.*

than the former two substrates. This sensitivity is particularly useful when measuring trace quantities of xylanase in such products as bread improver mixtures.

(c) Measurement of limit-dextrinase

Limit-dextrinase, also called debranching enzyme or pullulanase, is the enzyme which cleaves the 1,6-α-branch linkages in the amylopectin fraction of starch. This enzyme is thought to be the limiting activity in malt flour which dictates the degree of conversion of starch to fermentable sugars. Yeast cells readily absorb glucose, maltose and maltotriose, but are unable to absorb branched maltosaccharides. It is thought that the fermentability of wort (the malt extract) may be directly related to the level of limit-dextrinase in the malt. Microbial pullulanases, in combination with β-amylase are also finding increasing application in the starch-syrup industry in the production of high maltose syrups.

Measurement of limit-dextrinase in malt extracts using reducing sugar methods is difficult, time consuming, and at the best, semi-quantitative. The enzyme has to be partially purified before it can be assayed. However, with dye-labelled pullulan as substrate, activity in malt extracts can be assayed directly. A soluble, red-dyed pullulan is finding widespread application[14,15] in this area. A linear reaction curve in the range 0.1-1.4 absorbance units (at 510 nm) can be obtained, but the substrate is not as stable as would be preferred. An alternative substrate is dyed and crosslinked pullulan in tablet form (Limit-DextriZyme tablets) which is both more stable and more sensitive than red pullulan.

(d) Measurement of malt β-glucanase

Malt β-glucanase is a specific *endo* (1-3)(1-4)-β-D-glucanase which cleaves the 1,4-β-glycosidic bond of the 3-linked D-glucosyl units in mixed-linkage barley B-glucan. This enzyme is synthesised by barley during the malting process, but is very susceptible to thermal inactivation during kilning. Malt β-glucanase level is considered to be an important quality parameter of malt. With judicious mashing temperature profiles, this enzyme can be employed to partially depolymerise the mixed linkage β-glucan extracted from the malt (which causes filtration problems). This enzyme has traditionally been assayed by a viscometric procedure using barley β-glucan of intermediate viscosity as the substrate.[1] In recent years a method based on the use of the soluble azo-barley glucan,[8] has gained favour and is routinely used in many breweries and malt houses and by industrial enzyme manufacturers. Dye-labelled, crosslinked barley β-glucan in tablet form is currently under development.

(e) Measurement of endo*-arabinanase*

To obtain maximum juice extraction from apple and pear pulp, it is common practice to employ pectinase enzymes to destroy the gelatinous, water-binding properties of the pulp pectic polysaccharides. These pectic polysaccharides have a modified polygalacturonic acid backbone with highly substituted regions to which, 1,4-β-D-galactans and α-L-arabinans are attached. The arabinans have a 1,5-α-linked L-arabinan backbone to which single unit L-arabinosyl residues are linked α-1,3 and α-1,2. Pectinase enzyme mixtures are usually deficient in *endo*-1,5-α-L-arabinanase and thus high concentrations (about 3-5 mg/ml) of linear, 1,5-α-L-arabinan can occur in the juice concentrate. This arabinan self-associates and crystallises in the juice, leading to haze formation. The problem is most readily resolved by ensuring that adequate levels of *endo*-arabinanase (which depolymerises the linear arabinan) are present in the enzyme preparation employed. However, this has been complicated by the lack of a specific assay procedure for *endo*-arabinanase and even the non-availability of the basic polysaccharide substrate. This problem has now been resolved by the preparation of dyed, crosslinked, linear 1,5-α-L-arabinan. The linear arabinan was prepared by enzymic debranching of sugar-beet arabinan. Tablets of this dyed substrate[10] form the basis of a simple, very specific and highly sensitive assay for the measurement of *endo*-arabinanase.

(f) Measurement of cellulase and β-mannanase

Soluble, dye labelled substrates for the assay of cellulase (*endo*-1-4,-β-D-glucanase) and β-mannanase activities have been prepared by dyeing carboxymethyl cellulose and carob galactomannan, respectively, with Remazol brilliant Blue R dye. These substrates allow the specific measurement of *endo*-acting enzymes in the presence of *exo*-hydrolases. Dyed, crosslinked substrates for cellulase and β-mannanase have been developed and the performance of these in tablet form is currently being evaluated.

Conclusion

With continued pressure on cereal and fruit processors for increased throughput, improved yields and simpler processing formats, the efficient and effective exploitation of both endogenous and industrial enzymes, is imperative. This is only possible if rapid, reliable and specific enzyme assay procedures are available. Many of the procedures currently in use in the cereals and industrial microbiology industries are based on outdated and tedious technologies. There is an immediate requirement for further research, development and education in this area.

It is interesting to note that with the advent of genetic engineering, the technology available for the large-scale production of specific enzymes has actually outpaced the development of technologies for the accurate and reliable measurement of the enzyme activity.

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Cereal Chemistry Topics

Continued

Cereal Assay Kits

From November 1st the range of MegaZyme diagnostic kits currently available from Biocon (Aust.) Pty. Ltd. will be supplied directly from MegaZyme. These include kits for α-amylase, β-amylase, Barley β-glucan assay, malt β-glucanase assay, and glucose assay. MegaZyme will also offer a new "Starch Damage" assay kit and new enzyme test tablets for the measurement of α-amylase, β-xylanase, cellulase, *endo*-arabinanase, β-mannanase, limit dextrinase, malt β-glucanase, pure polysaccharides and oligosaccharide standards. *Contact:* MegaZyme (02) 872 3879. Fax: (02) 872 5367.