NEW DEVELOPMENTS IN THE MEASUREMENT OF α–AMYLASE, *ENDO*-PROTEASE, β–GLUCANASE AND β–XYLANASE

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1 INTRODUCTION

Over the past 8 years, we have been actively involved in the development of simple and reliable assay procedures, for the measurement of enzymes of interest to the cereals and related industries. In some instances, different procedures have been developed for the measurement of the same enzyme activity (e.g. α–amylase) in a range of different materials (e.g. malt, cereal grains and fungal preparations). The reasons for different procedures may depend on several factors, such as the need for sensitivity, ease of use, robustness of the substrate mixture, or the possibility for automation. In this presentation, we will present information on our most up-to-date procedures for the measurement of α–amylase, *endo*-protease, β–glucanase and β–xylanase, with special reference to the use of particular assay formats in particular applications.

2 α–AMYLASE

A range of substrates are available for the measurement of α–amylase, including:

1 Amylase HR Reagent (Ceralpha Method)[1]; containing end-blocked p-nitrophenyl maltoheptaoside in the presence of excess thermostable α–glucosidase.
2 Amylazyme[2] and Phadebas[R] Tablets; containing dyed and crosslinked starch.
3 Red Starch; a red-dyed, soluble starch substrate.
4 Beta–limit dextrin; for use in starch/iodine assay procedures (e.g. Farrand, International and SKB methods and FIA based procedures).
5 Soluble starch for use in reducing-sugar procedures.

Of the assay procedures available, the only one involving a defined substrate is the Ceralpha method[1] using Amylase HR Reagent. This reagent mixture contains the defined, modified oligosaccharide, "end-blocked p-nitrophenyl maltoheptaoside". The assay is very versatile and can be used to assay cereal, fungal and bacterial α–amylases. The principle of the Ceralpha assay procedure is shown in Figure 1. The replacement of the amyloglucosidase/yeast maltase enzyme mixture by thermostable α–glucosidase allows reagent to be used at temperatures up to 60°C and in the pH range 5.2 to 7.5 (formerly, it could only be used up to 40°C, and in the pH range 5.0 to 6.0). This assay procedure is extremely versatile and has been accepted as a standard by the International Association of Cereal Science and Technology (ICC). It has been successfully evaluated by the U.K. milling industry, and is currently replacing the

Farrand method, which has been in place since 1964. An interlaboratory evaluation by the American Association of Cereal Chemists (AACC) is currently being organised.

The Ceralpha method has been related to other procedures for the assay of α–amylase and equations relating these methods are given in Table 1.

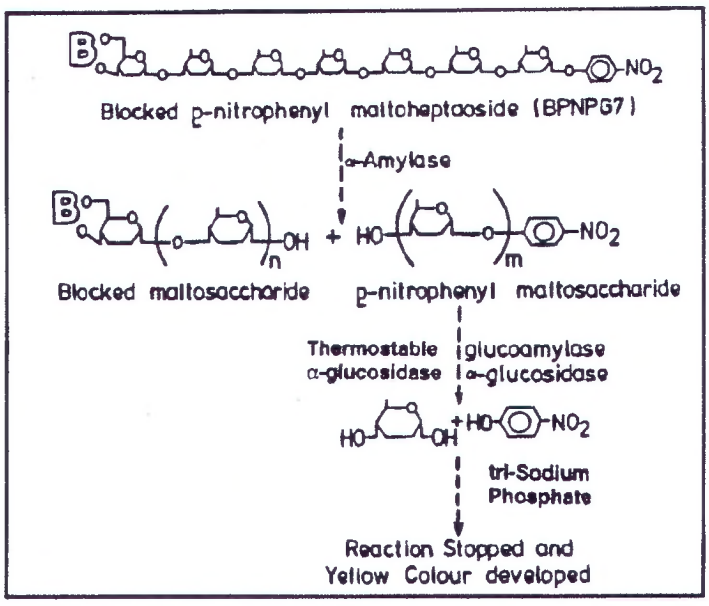

Figure 1. Ceralpha method for the measurement of α–amylase using either amyloglucosidase/yeast maltase mixture (Ceralpha Reagent) or thermostable α–glucosidase (Amylase HR Reagent).

Table 1. Correlation of the Ceralpha method to β–limit dextrin/iodine methods for the measurement of α–amylase.

Method	Source of Enzyme	Regression Equation
International Method (DU)	Malt	DU = Ceralpha (CU) x 0.30
SKB	Malt	SKB = Ceralpha (CU) x 0.57
(AACC)	Fungal	SKB = Ceralpha (CU) x 0.59
	Bacterial	SKB = Ceralpha (CU) x 1.80
Farrand	Wheat	FU = Ceralpha (CU) x 86–1.9
	Fungal	FU = Ceralpha (CU) x 69

Alternative procedures for the measurement of α–amylase involve the use of dyed, crosslinked amylose in tablet form (Amylazyme tablets[2]) or Procion Red dyed soluble starch. The major advantage of the Amylazyme substrate is that it is more robust (harder to contaminate), is more sensitive, and can be used in situations where Amylase HR can't be used. Assay formats based on this substrate are particularly useful for measuring trace levels of α–amylase in materials such as heat-treated flours and food products containing starch as a thickening agent, and for the measurement of α–amylase

in biological washing powders. The Amylazyme method has been adopted by AACC (Method 22–05) for the measurement of α–amylase in cereal flours and malts, following an extensive interlaboratory evaluation. Red Starch is a soluble dyed starch material for the measurement of α–amylase. This can be used in test-tube formats for α–amylase measurement, but it is also useful for detecting α–amylase in agarose and acrylamide gels.

One of the few automated methods for the measurement of α–amylase involves the use of the Scalar flow-injection analysis equipment. The substrate and assay procedure is a modification of the International (American Society of Brewing Chemists; ASBC) method that is based on the reaction of iodine with β–limit dextrin. Until recently, a β–limit dextrin material was available from Rank-Hovis. This material was prepared by treatment of starch with soybean β–amylase. The substrate as supplied was a mixture of β–limit dextrin and maltose in approximately equal proportions. Since Rank Hovis decided not to continue production of this material, Megazyme was approached as a possible alternative manufacturer. The material now produced and supplied by Megazyme, behaves in the assay procedure in a very similar way to the Rank-Hovis product, however, essentially all of the maltose has been removed by ultrafiltration. Consequently, the concentration of the Megazyme β–limit dextrin used in α–amylase assays (1 %) is half the concentration recommended for the Rank-Hovis material. With the Scalar flow-injection analysis procedure for the measurement of α–amylase, the values obtained are essentially identical with the values obtained with the ASBC method (using a β–limit dextrin produced from a special starch provided by ASBC).

3 β–XYLANASE

Several substrates and assay procedures are available for the measurement of β–xylanase (*endo*-1,4-β–xylanase) [2] in enzyme materials and food and feed products, and some of these are summarised in Table 2.

Table 2. Substrates for the assay of β–xylanase.

Substrate	Nature	Assay Procedure
Wheat arabinoxylan	soluble	Reducing-sugar
Wheat arabinoxylan	soluble	Viscometric
Xylazyme AX Tablets	gel particles	Chromogenic substrate
Azo-wheat arabinoxylan	soluble	Chromogenic substrate
Azo-xylan (oat spelts)	soluble	Chromogenic substrate
Azo-xylan (birchwood)	soluble	Chromogenic substrate

In creating standard curves for the various dyed substrates, the β–xylanase used is first standardised in International Units. The Nelson-Somogyi reducing-sugar method is used with wheat arabinoxylan as substrate, and one Unit of activity is defined as the amount of enzyme required to release one micromole of reducing-sugar equivalents (as xylose)

per minute, under the defined assay conditions of temperature and pH. The Nelson-Somogyi reducing-sugar method is one of the few reducing-sugar methods, which gives a stoichoimetric colour response with homologous oligosaccharides of increasing degrees of polymerisation.

Although reducing-sugar methods are useful for standardising the activity of relatively pure *endo*-β–xylanase, they cannot be used for samples containing high levels of reducing sugar, or for preparations with high levels of other enzymes active on the substrate (e.g. β–xylosidase and α–L-arabinofuranosidase). In such cases, viscometric methods, or methods employing dyed xylan substrates must be used. Viscometric assays are highly specific for *endo*-enzymes, particularly if a high viscosity substrate is used. For β–xylanase, the substrate of choice is wheat arabinoxylan. Such assays can be sensitive, specific and accurate, however, unless an automated viscometer is available, the assays are tedious and time consuming. Results are most meaningful if several measurements are taken over an incubation period, and inverse reciprocal viscosity values are determined and plotted against incubation time.

Several chromogenic substrates are available for the specific measurement of *endo*-β–xylanase. Of these, Azo-wheat arabinoxylan (soluble) and Xylazyme AX (gel particles in tablet form) are the most useful and versatile substrates. A comparison of the sensitivity of various soluble chromogenic xylan substrates is given in Figure 2. Of these, Azo-wheat arabinoxylan is the most sensitive. Assays employing Xylazyme AX tablets are approximately 5-times more sensitive than those using Azo-wheat arabinoxylan, consequently, where sensitivity is the major concern, the Xylazyme AX tablets are the substrate of choice.

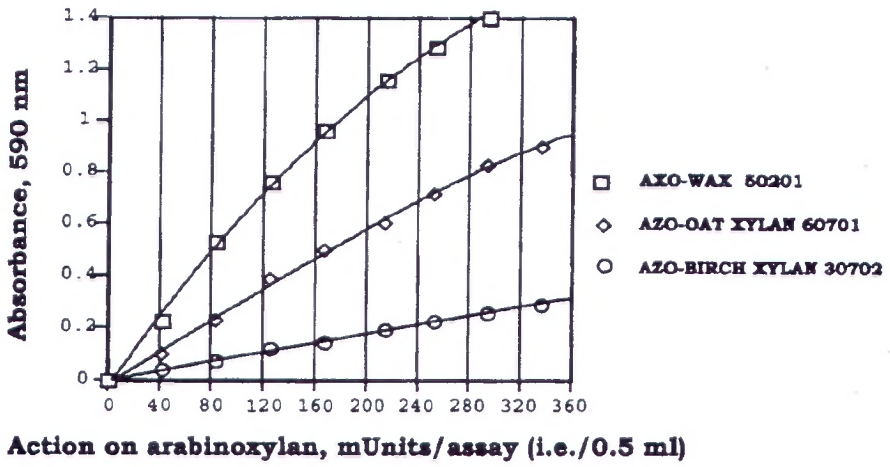

Figure 2. Standard curves relating enzyme activity of pure A. niger xylanase to colour release from Azo-wheat arabinoxylan, Azo-xylan (oat) and Azo-xylan (birchwood).

The measurement of xylanase in industrial enzyme preparations is relatively straight-forward. However, problems arise when measuring trace levels of enzymes in animal feeds or bread improver mixtures. These problems include adsorption to feed

components, inactivation during pelletting and inhibition by specific xylanase inhibitors (such as those identified in wheat flour). The best approach to get a true estimate of the level of enzyme in the feed is through recovery experiments. Basically, a known quantity of a particular xylanase preparation is added to a slurry of the feed, and the recovery of activity is determined using a suitably sensitive substrate, such as Xylazyme AX tablets or Azo-wheat arabinoxylan.

4 β–GLUCANASE AND CELLULASE

Various substrates are available for the measurement of *endo*-1,4-β–glucanase (cellulase) and *endo*-1,3;1,4-β–glucanase (lichenase, malt β–glucanase) [2] as shown in Table 3. Each of the substrates listed has particular advantages and limitations.

Table 3. Substrates for the assay of cellulase and β–glucanase.

Substrate	Nature	Assay procedure
CELLULASE:		
CM-Cellulose 7M	soluble	Reducing sugar or viscometric
CM-Cellulose 4M	soluble/gel	Reducing sugar
Azo-CM-Cellulose	soluble	Chromogenic substrate
Azo-Barley Glucan	soluble	Chromogenic substrate
Cellazyme C Tabs	gel particles	Chromogenic substrate
Cellazyme T Tabs (tamarind xyloglucan)	gel particles	Chromogenic substrate
Beta–Glucazyme Tabs	gel particles	Chromogenic substrate
1,3;1,4-BETA–GLUCANASE;		
Azo-Barley Glucan	soluble	Chromogenic substrate
Beta–Glucazyme Tabs	gel particles	Chromogenic substrate

Pure barley β–glucan is useful for the assay of purified cellulase and β–glucanase in reducing-sugar assays. It is also used in viscometric assays. The official method of the Institute of Brewing for the assay of malt β–glucanase employs barley β–glucan in a viscometric procedure. The enzyme is mixed with the substrate and viscosity measurements are taken at several times over about 30 min. Inverse reciprocal viscosity (IRV) values are calculated and activities are determined from plots of IRV against incubation time. The assay is relatively accurate and reproducible, but very time consuming; analysis of 10 samples takes a single operator about 2 days. An alternative procedure involves the use of Azo-Barley Glucan[3]. With this substrate and assay procedure, 10 samples can be extracted and analysed in about 1 hour. With the Azo-Barley Glucan method, absorbance values can be converted to International Units of activity through a standard curve, or alternatively can be directly converted to IRV units. A curve relating enzyme activity determined with the Azo-Barley Glucan method (substrate lot 60602) to IRV units for several malt samples is shown in Figure 3. A range of dyed polysaccharide substrates have been developed for the measurement of

cellulase, including Cellazyme C (dyed, crosslinked HE-cellulose), Cellazyme T (dyed, crosslinked xyloglucan) and Beta–Glucazyme (dyed, crosslinked β–glucan). At first glance, one would conclude that the best substrate to assay for enzymes active on barley or oat β–glucan is the Beta–Glucazyme tablets. However, if sensitivity is the major concern, as is the case for the measurement of trace levels of cellulase in animal feeds, the preferred substrate is Cellazyme T tablets. (see Figure 4).

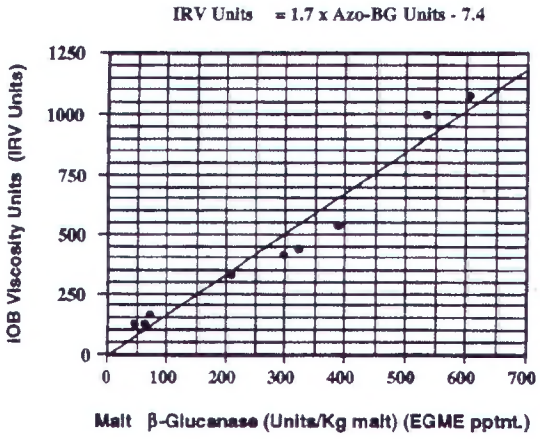

Figure 3. Curve relating IRV units (IOB method) to malt β–glucanase activity determined with the Azo-Barley Glucan method (substrate lot 60602).

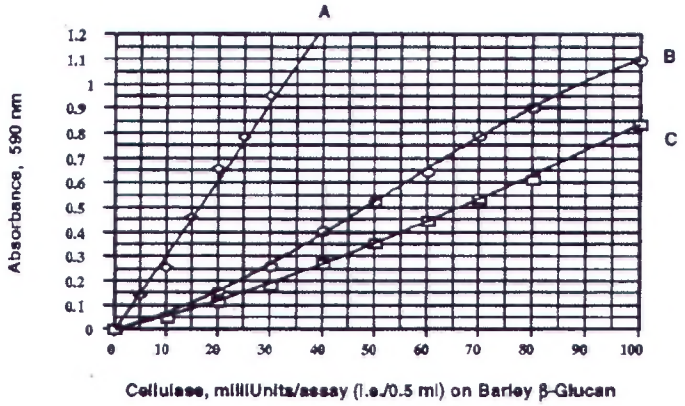

Figure 4. Standard curves relating enzyme activity of pure T. viride cellulase to colour release from Cellazyme C (C), Cellazyme T (A) and Beta–Glucazyme (B) tablets.

When sensitivity is not a constraint, Azo-CMC is the preferred substrate. Firstly, because the level of salt in the sample does not affect the assay, and secondly, the standard curves relating enzyme units (International Units) to absorbance increase were found to be very similar for a range of cellulases. Cellazyme C tablets find widespread use in the denim industry for standardisation of cellulases used in creating the "stone-washed" effect.

5 PROTEASE

Two commonly used substrates for the assay of *endo*-protease are cow hide azure and Azo-Casein. Cow hide is essentially collagen. We have prepared a dyed, crosslinked collagen product (AZCL-collagen) as an alternative to cow hide azure. The major advantage of AZCL-collagen is that it is less fibrous and can thus be incorporated into tablets (Protazyme OL), which greatly increases the ease of use of this substrate.

However, in comparing a wide range of *endo*-proteases on Protazyme OL tablets (AZCL-collagen) to action on Protazyme AK tablets (AZCL-casein), it was found that the relative rates of hydrolysis of the two substrates were similar. Consequently, since the standard curves with Protazyme AK are more linear than with Protazyme OL, then Protazyme AK is the substrate of choice.

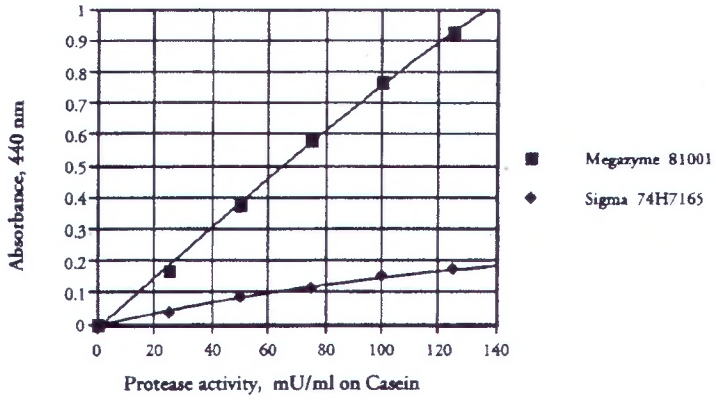

Figure 5. Standard curves for Subtilisin A on Azo-casein (Megazyme, lot 81001 and Sigma, lot 74H 7165). The Subtilisin A was first standardised on casein using tyrosine as the standard.

A disadvantage of the Protazyme tablets is that disintegration of the tablets requires agitation. Thus, a stirrer bath arrangement is required. An alternative substrate is soluble Azo-Casein. Azo-Casein has been commercially available for many years, but most commercial preparations are poorly dyed, and incompletely soluble, resulting in limited sensitivity and poor linearity of the standard curve. We have re-evaluated the dyeing of casein with sulphanilic acid, and have optimised this reaction to give a highly dyed material which is a very effective substrate for *endo*-proteases. In Figure 5, standard curves for Subtilisin A on Azo-Casein from Megazyme (lot 81001) and from Sigma Chemical Co. (lot no. 74H7165), are compared. It is obvious that the Megazyme product is superior in terms of sensitivity and linearity of the standard curve. The sensitivity of *endo*-protease assays using Azo-Casein (Megazyme lot no. 81001), is about one third of that using Protazyme AK tablets. Azo-Casein is a good, non-selective substrate for the assay of *endo*-protease activity. Regression equations for a number of *endo*-proteases on Azo-Casein are given in Table 4.

Table 4. Regression equations for several proteases on Azo-Casein (lot 81001).

Papain (from *Papaya* latex): Protease (milli-Units/mL) = 270 x Absorbance (440 nm) + 7;	R = 0.99
Bromelain (from pineapple stem): Protease (milli-Units/mL) = 460 x Absorbance (440 nm) - 13;	R = 0.99
Ficin (from figs): Protease (milli-Units/mL) = 190 x Absorbance (440 nm) + 3;	R = 0.99
Subtilisin A (from *Bacillus licheniformis*): Protease (milli-Units/mL) = 130 x Absorbance (440 nm) + 4;	R = 0.99
Proteinase K (from *Tritirachium album*): Protease (milli-Units/mL) =140 x Absorbance (440 nm) - 4;	R = 0.99
Fungal protease (*A. niger*; from Sigma Chemical Co.): Protease (milli-Units/mL) = 146 x Absorbance (440 nm) - 4;	R = 0.99

The linear range for each of the proteases was essentially 0.1 to 1.0 absorbance units.

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