FEED MIX SPECIAL SERIES

- enzymes - nature's catalysts
- triggering the breakdown of nutrients
- enzymes in action
- tackling the phosphate burden
- optimising the response
- beyond the horizon the future for enzymes

ENZYMES - NATURE'S CATALYSTS

OPTIMISING THE RESPONSE

Measurement of trace levels of enzymes in feeds is difficult due to problems of extracting the enzyme from the feed

A fine balance exists between enzyme activity and the adverse effects associated with feed processing. Accurate estimation of enzyme activity in the feed is a pre-requisite to optimising the response.

By Dr T Acamovic and Dr B V McCleary

Advances in biotechnology to allow the commercial production of enzymes now allows benefits of such enzymes to be realised by their addition, at the correct levels, to the diets of pigs and poultry. Enhanced productivity, as well as improved welfare and hygiene, is the outcome.

Removal of feed constraints

A major advantage in using enzyme supplementation in pig and poultry diets is that ingredients that are otherwise not used, or included only at low levels, may be utilised to a greater extent without loss of performance and at lower costs than conventional ingredients. The use of these other ingredients to substitute for ingredients such as meat and bone meal which have been removed from some markets, is made more likely by the improvements which may be made by enzyme treatment. Ingredients such as cottonseed meal, rapeseed meal and sunflower seed meal appear to respond very well to enzyme treatment (Bento, Acamovic and Bedford, unpublished data) and others may respond equally well or better. Enzyme treatment of tanniniferous feeds (faba beans) has substantially improved the digestibility of nutrients, where improvements were greater at higher tannin content (Salawu *et al*, 1995). Nutrient availability of lupins and peas in poultry diets has also been improved (Ferraz de Oliveira and Acamovic, 1995; Annison *et al*, 1995; Cowan and Korsbak, 1996).

Nutritive value of feedstuffs

Apparent nutritive value is closely associated with the content of any anti-nutritional or non-nutritional factors in the feedstuffs since these can increase endogenous losses from the animal. Some antinutrients such as beta-glucans and some lectins are resistant to digestion or cannot be digested by the endogenous enzymes of the animal. They may inhibit activity of the endogenous enzymes, cause increased secretion of endogenous enzymes, or, upon being attacked by endogenous enzymes, produce compounds which do not benefit the animal. Nutrients may also be resistant to digestion because of their structure. For example, some legume proteins are more resistant to digestive enzyme attack than others, and more resistant than proteins from other sources such as animal and cereal proteins. Frequently, the various carbohydrate fractions in feeds cannot be digested by monogastrics and in some instances the compounds produced by enzymatic degradation can be detrimental. Aspects of these have been discussed in previous papers in this series.

Enzyme systems in use

The enzyme systems currently being used commercially and investigated include a wide variety of carbohydrases,

proteases, phytases and lipases to enhance protein, energy and mineral digestion. The most effective and thoroughly studied of the supplemental enzymes are the beta-glucanases which degrade large beta-glucan molecules to smaller moieties and which improve metabolisable energy and nutrient utilisation of barley-based diets for poultry. Similar effects have been observed for the absorption of nutrients in diets of chicks fed wheat-based diets where the main carbohydrates restricting absorption were arabinoxylans (Mul and Bonte, 1995).

Scope for cellulases

Since monogastric animals do not digest cellulose (a glucose polymer) to any great extent, it seems that there is plenty of scope for the development of fast acting, potent cellulases so that fibrous waste material such as straws and haulms from cereals and legumes could be utilised as a source of energy for monogastrics. The production and use of such cellulases is under study (Clarkson and Morgan, 1995).

While Classen (1996) stated that complete hydrolysis of polysaccharides to monomers was not necessary for improved performance, in the case of glucose polymers it is desirable since this carbohydrate can be used very efficiently as a source of energy.

Optimal activity

A problem associated with enzyme supplementation of monogastric diets is the relatively short transit time of feeds through the gastro-intestinal tract. Thus the enzymes need to be present in sufficient quantity, act rapidly and be resistant to the conditions that exist in the gastro-intestinal tract. In this instance, addition of more enzyme is not necessarily the best method by which to enhance performance. Indeed, in some cases the effect of increased additions may be detrimental; thus the optimal level of enzyme activity, rather than increased concentration, is required.

Some work has shown that enzyme supplementation of rapeseed meal based diets improves nutrient retention (Bedford, 1995) while others demonstrate that the enzyme effect can be overcome by the presence of large quantities of rapeseed meal or other dietary ingredients (Aboosadi *et al*, 1996; Khattack *et al*, 1996; Wallis *et al*, 1996).

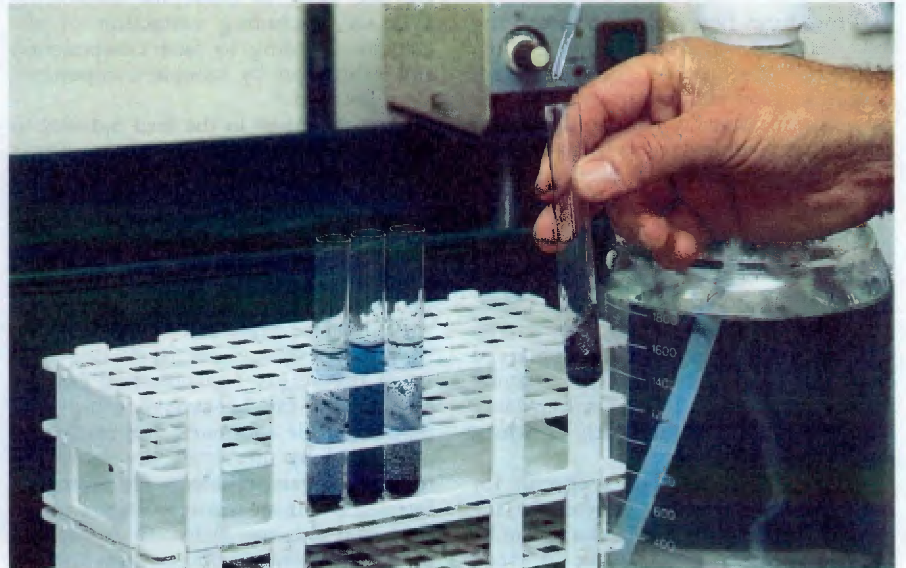
There is often a synergism between various enzymes in a diet (although this is not always so). In some cases the content of antibiotic, and thus the microflora profile within the gut, in association with enzymes, can have beneficial effects (Allen *et al*, 1996).

Factors influencing activity

The optimal activity of the enzyme in the diet is influenced by dietary pre-treatment, pH in the gastro-intestinal tract, the length of the gastro-intestinal tract and the degree of hydration and body temperature of the animal (chicken higher than pig) as well as the susceptibility of the exogenous enzyme to endogenous enzyme attack.

Other factors which influence the optimal activity include the concentration of product due to enzyme hydrolysis, endogenous enzyme activity/concentration, and the type of ingredient (Cowan and Korsbak, 1996).

Each enzyme must be considered individually, and appropriate recovery experiments performed



Substrate differences and inclusion rate

Work at Aberdeen shows that supplementation of lupin-based diets with carbohydrases at the same level influences amino acid and protein digestibility in different ways, depending on the type of lupins used (Ferraz de Oliveira and Acamovic, 1996; Ferraz de Oliveira *et al*, 1996). From this it can be assumed that the carbohydrate, glycoprotein and protein compositions and structures differ sufficiently between the two cultivars to give different responses *in vitro*.

Other work with poultry indicates positive responses in metabolisable energy and dry matter digestibility occurring in lupins when the enzyme level is increased (Ferraz de Oliveira and Acamovic, 1995) and this has also been shown to be the case with wheat-based diets fed to poultry (Mul and Bonte, 1995).

Withstanding feed processing

Exogenous enzymes added to monogastric diets must be able to function adequately after they are added to the dietary ingredients, often at very low concentrations (mg/kg) and under adverse conditions of heat and other chemical stress.

During feed processing, enzyme preparations are subjected to hostile conditions which may reduce their activity. Bearing in mind that heat treatment is used to denature and reduce trypsin inhibitor and lectin activities in various ingredients, it is not surprising that the heat generated during the processing of feeds could reduce supplemental enzyme activity.

It is also not unexpected that different enzymes vary in their susceptibility to processing. Thus the enzyme activity added to diets and ingredients may not be what is present after processing. In such a complex system there is a fine balance to be achieved; one which is influenced by the enzyme activity and by possible adverse effects associated with feed processing. However, difficulties exist in quantifying the activities of the supplemental enzymes present in feedstuffs.

Measuring enzyme content of feed

The measurement of trace levels of enzymes in such heterogeneous mediums as animal feeds is difficult. With each enzyme, many of the problems will be similar; low levels of activity, extraction problems and

Measurment of feed enzyme activity is facilitated by the development of rapid, specific and sensitive assay procedures

inactivation during pelleting and following extraction from the feed. With some enzymes, the problems will be more pronounced; variable extraction of different xylanases from feeds and differences between different feed mixtures.

It is essential that each enzyme is considered individually and that appropriate recovery experiments are performed. All of this work is facilitated by the development of rapid, specific and sensitive assay procedures.

Measurement of polysaccharide *endo*-hydrolases

Several procedures have been developed for the specific measurement of polysaccharide *endo*-hydrolases in the presence of *exo*-polysaccharidases and glycosidases. These include:

- viscometric assays using pure polysaccharides (Bathgate, 1979)
- assays based on soluble, dyed polysaccharides (McCleary and Shameer, 1987)
- assays using insoluble (crosslinked) dyed polysaccharides (McCleary, 1995)
- methods which measure interaction of a native polysaccharide with a second compound eg β-glucan with congo red (Martin and Bamforth, 1983)
- assays using well defined oligosaccharides with a chromophore attached eg "end-blocked" ρ-nitrophenyl maltoheptaoside (McCleary and Sheehan, 1987)

β-xylanases difficult to measure

One of the most difficult enzymes to measure in feeds is β-xylanase. This is due to several factors, such as the low levels added to the feed, binding of some xylanases to feed components, and inhibition by soluble feed components.

The enzyme activity added to diets may not be what is present after processing

Procedures best suited to measuring trace levels of xylanase added to chicken feeds are viscometric, or use dyed, crosslinked wheat arabinoxylan. These two systems have a similar degree of sensitivity. Soluble dyed xylans and dyed arabinoxylans can also be used, but they are much less sensitive.

Viscometric assay for β-xylanase

While viscometric assays are tedious, good linearity between enzyme activity and viscosity change can be obtained over a wide range of enzyme concentrations (10-fold), as long as a high-viscosity substrate such as wheat arabinoxylan is used.

Because this procedure is specific for *endo*-xylanase and is not affected by the presence of sugars in the extract, it can be used to measure *endo*-xylanase in crude feed samples. However, extraction of enzyme from the feed, stability of the extracted enzyme, and interference by enzyme inhibitors in the extract will influence the accuracy of the result.

Assays using dyed, crosslinked polysaccharides

In the preparation of dyed, crosslinked xylans or arabinoxylans as substrates for β-xylanase measurement, several parameters are important, including the purity and the molecular size of the polysaccharide, and the degree of natural substitution.

With low molecular weight material it is not possible to produce stable, dyed and crosslinked gel particles. To obtain a consistent quality substrate, a source of xylan, such as wheat flour, in which the substrate is arabinoxylan, must be used.

Assays using tablets, which have the same sensitivity and specificity as viscometric assays, have the added advantages of simplicity and rapidity. The principle of this and other assays using dyed and crosslinked polysaccharide substrates is outlined in *figure* 1.

Since xylanases from different sources, and even different forms of the enzyme from the same source have different action patterns, standard curves relating enzyme activity (by a method which measures bonds broken) to colour released upon hydrolysis will vary. This need not be a problem in standard practical applications, because in most situations a single preparation is being evaluated, either in fermentation situations or in feed applications.

β-glucanase and cellulases

Many of the problems experienced in measuring β-glucanases in animal feeds are the same as those for measuring xylanase, including extraction of the enzyme (binding to feed components) and inhibition by soluble components from the feed.

Enzymes used in the feed industry to depolymerise mixed-linkage beta-glucans from barley or oats include *endo*-1,4-β-D-glucanase (cellulase) and *endo*-1,3:1,4-β-D-glucanase (β-glucanase). Both of these enzyme groups can be assayed using barley β-glucan or dyed barley β-glucan. Cellulase enzymes can also be assayed with cellulose based substrates.

Endo-Beta-glucanases (including *endo*-1,4-β-D-glucanase) in industrial enzyme preparations can be assayed using pure polysaccharide substrates. The reaction is monitored by measuring the release of reducing sugars. Problems are caused by interference from reducing sugars in the feed, and non-specificity - it is essential to use an assay specific for *endo*-activity.

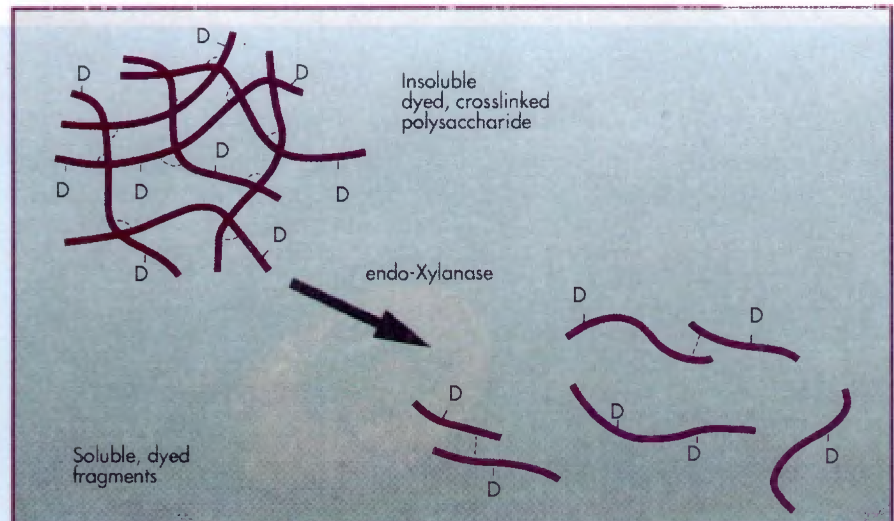


Figure 1 *Principle of assay of polysaccharide endo-hydrolases using dyed, crosslinked substrates*

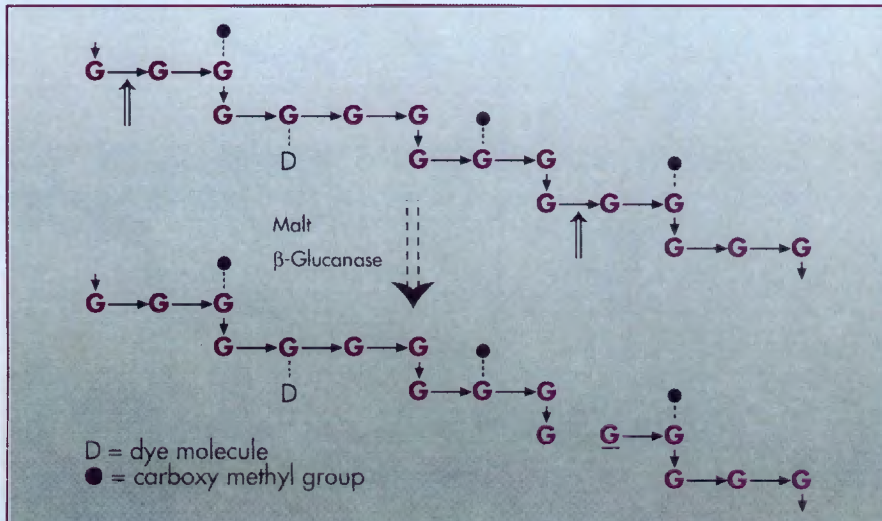


Figure 2 *Principle involved in the assay of polysaccharide endo-hydrolases using soluble, dyed polysaccharides*

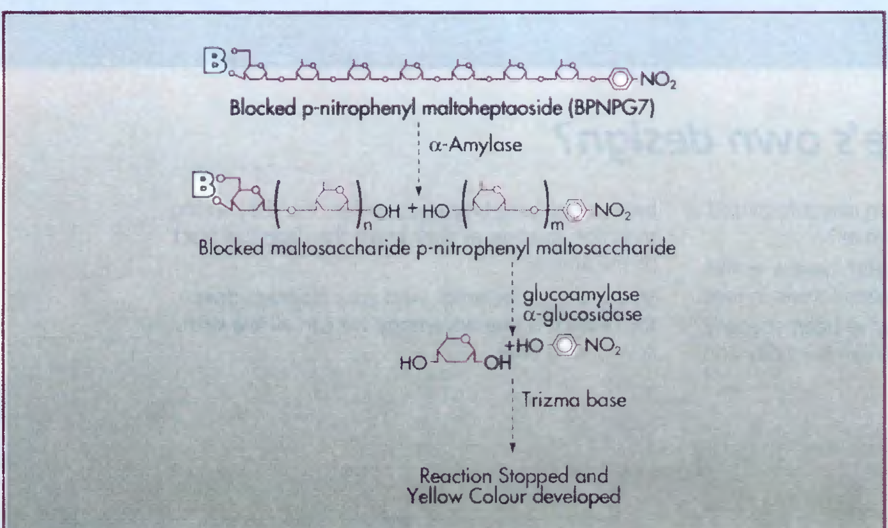


Figure 3 *Principle involved in the assay of α-amylase using end-blocked p-nitrophenyl maltoheptaoside*

Alternative assays use dyed β-glucan, or dyed cellulose derivatives. The soluble substrate, azo-barley glucan (McCleary and Shameer, 1987) is now widely used in the malting, brewing and animal feed industries. In principle, this assay involves incubating the enzyme with a high molecular-weight dyed polysaccharide under defined conditions (*figure* 2).

Alternatively, insoluble, dyed polysaccharide tablets can be used; these contain barley β-glucan or cellulose as the primary substrate.

Alpha-amylase

There is some interest in the addition of α-amylase to feeds to improve digestibility. Several good methods are available for the measurement of this activity and these include those employing dyed-crosslinked starch or amylose and those using blocked p-nitrophenyl maltosaccharides. (McCleary and Sheehan, 1987).

Assays based on the use of p-nitrophenyl maltosaccharides generally employ an "end-blocked" nitrophenyl maltosaccharide in the presence of excess quantities of the *exo*-acting enzymes, amyloglucosidase and α-glucosidase. The blocking group prevents hydrolysis of the substrate by these enzymes.

The principle of this assay is shown in *figure* 3. When *endo*-acting α-amylase cleaves the maltosaccharide, the *exo*-acting enzymes instantaneously hydrolyse the nitrophenol maltosaccharide reaction-product to glucose and free p-nitrophenol. The reaction is terminated and the colour developed by the addition of a weak alkaline solution.

This is a simple and very specific assay for the measurement of α-amylase, and can be used to specifically measure the enzyme in the presence of other starch-degrading enzymes such as amyloglucosidase and β-amylase. The assay is ideal for measuring α-amylase in industrial microbial preparations, and can also potentially be used to measure this enzyme in feed preparations. The only limitation may be sensitivity. If the levels of activity are too low to be accurately measured by this method, then assay formats employing dyed, crosslinked substrates in tablet form should be used.

α-Galactosidase enzymes

Another group of industrial enzymes which are finding application in the feed industry are the α-galactosidases. These act on the antinutritional, raffinose-series oligosaccharides producing sucrose and free galactose. For these enzymes to be effective, they must effectively hydrolyse raffinose, stachyose and verbascose, and they must be able to act under conditions of limited water availability.

α-Galactosidase can be readily assayed using p-nitrophenyl α-D-galactoside as the substrate. Assays based on this principle are simple, specific and are not affected by reducing sugars in the feed. For a particular α-galactosidase the relative rates of hydrolysis of p-nitrophenyl α-D-galactoside, raffinose, stachyose and verbascose should be determined to ensure that the enzyme being used is actually effective on the component of interest. Activity of the purified enzyme on the raffinose-series oligosaccharides can be determined using a reducing-sugar assay or by the measurement of galactose released using galactose dehydrogenase.

Protease measurement still unsatisfactory

At present there are no good procedures for measuring residual proteolytic activity in feeds. Techniques that are used for protease determination cannot be used with crude feed extracts and/or they do not have the required sensitivity. There is scope for developing fluorimetric substrates and for improving assay formats employing chromogenic substrates such as Azo-casein or Azo-albumin. This is an area in which we are actively involved.

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