METHOD OF ANALYSIS FOR FEED ENZYMES: METHODOLOGICAL PROBLEMS?

ALAIN M. SABATIER and NEVILLE M. FISH
Rhone-Poulenc Animal Nutrition, 42, Avenue Aristide Briand BP 100,
92164 Antony Cedex, France
Phone: +33, (1) 46747106
FAX: +33, (1) 46747267

Primary Audience: Nutritionists, Laboratory Personnel

SUMMARY

Enzyme products currently on the market used as processing aids to enhance feed raw materials have the effect of breaking down macromolecules such as hemicellulose, or proteins. Since enzyme users must know the activity of the enzyme product in order for them to rationally formulate their diets, it is necessary to assay enzymes in the feed.

Several measurement methods exist for the analysis of enzymes in feed. Are there methodological problems in measuring enzyme activity that could restrict their utilization? Could the choice of an enzyme for a specific application be based on the number of units of enzyme activity?

In order to answer these questions, we shall clarify what an enzyme is and how its activity is measured: 1) enzymes function only through their catalytic action and an enzyme is specific for a substrate and catalyzes a specific reaction under defined conditions, 2) enzyme activity is measured by different methods, for which substrate quality is of the utmost importance, 3) there is a defined method for each product as a function of its origin and we have to adapt the method of analysis to each feed.

Key words: Analytical procedure, enzymes, feed

INTRODUCTION

Feed manufacturers now use enzymes as processing aids in order to enhance feed raw materials, primarily cereals. With the exception of phytase, enzyme products currently on the market have the effect of breaking down macromolecules such as hemicellulose, or proteins. The reaction is a hydrolysis, a depolymerization of these macromolecules, which improves the energy value and the digestibility of the feed and reduces pollution.

Enzyme users have to know the activity of the enzyme product in order to rationally formulate their diets. It is thus necessary to assay enzymes in the feed, a necessity which is coupled to the regulatory requirements being set up in the EU. Are there methodological problems in measuring enzyme activity that could restrict their utilization? In order to

2 To whom correspondence should be addressed
answer this question, we must clarify what an enzyme is and how its activity is measured.

**WHAT IS AN ENZYME ACTIVITY?**

Enzymes are products of biological origin that catalyze the biochemical reactions involved in cell life. Enzymes are proteins of high molecular weight (between 10,000 and 500,000 daltons), precipitated by alcohol, acetone, and ammonium sulphate. Like all proteins, they are sensitive to the physicochemical environment, variations in which may modify their activity.

Enzymes function only through their catalytic action, in contrast to other feed additives such as vitamins, growth promoters, or amino acids, whose efficacy is the result of their metabolism. Enzymes can catalyze the reaction of a large quantity of material, the substrate, in a short time. It is documented that 1 mole of enzyme may react 1000 to 10,000 time per sec with the substrate (invertase may catalyze the hydrolysis of 10 times its weight of sucrose per sec), and the catalyst is left chemically intact at the end of reaction. The fast rate of the reactions catalyzed by enzymes is due to the high affinity of the enzyme for the substrate, which is reflected in the binding of the substrate on the enzyme and the release of the converted product.

An enzyme is specific for a substrate and catalyzes a specific reaction under defined conditions. Chemical, as opposed to biochemical catalysts, acts on a wider range of components. Chemical reactions are often non-specific – specificity is obtained by selecting the appropriate reactants and their concentration. In contrast, an enzyme is specific of a given reaction, on a defined substrate (e.g. a cellulase will catalyze a reaction on cellulose, a β-glucanase on β-glucans, a xylanase on arabinoxylan, an amylase on starch, a protease on protein) even in the presence of other reactants. Furthermore, the substrate needs to be of a defined quality. For example, similar results are obtained from xylanase with xylan substrates extracted from birchwood or from wheat (Table 1).

The above results, obtained with xylanase from four different microorganisms, demonstrate well that the activity ratio may double depending on the source of substrate (birchwood or wheat). The interactions between enzyme, substrate, and product(s) are sensitive to the physicochemical conditions of the reaction, which may modify the reaction rate.

A catalytic action under defined physicochemical conditions is only possible through the binding of enzyme to substrate. The binding of enzyme to substrate depends on protein conformation, which is affected by pH and temperature. These factors also directly influence reactivity. In the example of endo-1,3(4)-β-glucanase activity from *Penicillium funiculosum*, the activities are different when measured at pH 3 or pH 5, or when the assay is performed at 40°C or 60°C (Figures 1 and 2).

It is clear that verification of enzymes activity requires the conditions supplied by the manufacturer. The conditions under which enzymes activities are measured need to be taken into account before interpretation of the analytical results, especially when comparing the activity of different enzymes. Comparison of two products from different microbial sources depends to a great extent on the conditions of the assay. Moreover, these activity curves are obtained in well-defined *in vitro* assay conditions which are not exactly representative of the *in vivo* animal conditions (pH, ionic strength, chemical environment, substrate concentration, quality of the substrate, etc.). Consequently, it seems very difficult to

**TABLE 1. Endo-1,4-β-xylanase activities on two xylan substrates**

<table>
<thead>
<tr>
<th>SOURCE OF Xylanase</th>
<th>ENZYME ACTIVITY (U/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Wheat Arabinoxylan</td>
</tr>
<tr>
<td><em>Aspergillus niger</em></td>
<td>490</td>
</tr>
<tr>
<td><em>Penicillium funiculosum</em></td>
<td>5,840</td>
</tr>
<tr>
<td><em>Trichoderma longibrachiatum</em></td>
<td>88,420</td>
</tr>
<tr>
<td><em>Trichoderma viride</em></td>
<td>149,000</td>
</tr>
</tbody>
</table>

*Activity measured using a DNS based assay method, under the same conditions of pH and temperature, and with the same concentration of substrate*
Enzyme activity is a measure of reaction rate, or the rate of transformation of substrate (i.e., the quantity of substrate transformed or product formed per unit time). Reaction conditions are fixed, since the rate or reaction is proportional within certain limits to the quantity of enzyme. An excess of substrate is present to minimize changes in conditions during the assay, and measurements are made of the quantity of substrate reacted, or product formed, per second. The activity of the enzyme, the initial rate of reaction, is expressed in units of enzyme per unit of weight or volume of preparation.

For example:

1 unit of phytase activity is the quantity of enzyme needed to release in 1 min, under defined conditions, 1 nmole of inorganic phosphate from sodium phytate;

1 unit of endo-1,3(4)-β-glucanase is the quantity of enzyme needed to release in 1 min, under defined conditions, 1 mg maltose equivalents from barley β-glucan;

1 unit of endo-1,4-β-xylanase is the quantity of enzyme needed to release in 1 min, under defined conditions, 1 mg maltose equivalents from birchwood xylan.

The definition of the physico-chemical conditions under which the measurements are made is essential to the definition of the activity of each enzyme. As the reaction conditions vary in accordance to the origin of the enzyme, the different units used cannot be compared.

Enzyme activity is measured by different methods for which substrate quality is of utmost importance. Several analytical methods are used, based upon a hydrolysis reaction of the substrate. The substrate, a non-starch polysaccharide (NSP), is made of polymeric branched chains. Enzymes having endo activities introduce breaks inside the macromolecule. The objective of the analytical method is to measure this endo activity as it reflects the depolymerization of the NSP's, the effect sought in animal feed applications.

To measure these activities several analytical methods are used. The more widely used are the colourimetric and the viscometric methods, which are based on the measurement of the quantity of product released during the enzyme reaction on a defined substrate.

One type of method makes use of the release of reducing sugars from non-starch polysaccharide substrates like barley β-glucan or carboxymethyl cellulose. The analysis consists of a measurement of the quantity of reducing sugars released during the enzymatic reaction in a set time, and the results are expressed in the form of a reaction rate (quantity – μmoles or g – reducing sugars released per unit time).
When measuring the activity in a complex environment like feed, the results may be compromised by other existing activities like cellubiohydrolases and β-glucosidases, which partially hydrolyze the β-glucan or xylan substrates and release reducing sugars, as a result of their exo activity. These methods, by their nature, are therefore used more commonly to measure exo activities. The environment may contain other reducing agents as well as minerals which will disturb the analysis and give falsely high readings.

Thus, these methods are not very suitable when measuring enzyme activities in feed, unless correlated with results obtained by another method that will take into account any interferences such as reducing products, and the exo activities. It follows that in the area of animal nutrition, it is necessary to use methods that account for the endo activities and that eliminate the interferences of the environment on the analysis. These conditions are satisfied when using either particular substrates, or a method that measures a specific physical property, not the release of a product.

The measurement of dye release from a defined substrate coupled to a dye (substrate chromophore) works on specified substrates, molecules chemically similar to the natural substrate. The analysis consists of a measurement of the release of the chromophore, a measure of endo activity, in a defined time interval under the specified assay conditions. The unit of activity is the quantity of enzyme needed to hydrolyze the substrate in order to release in a defined time, under specific conditions, soluble oligomers producing a given absorbance (OD units) at a specific wavelength.

The major concern with these techniques is the quality of the substrate. The substrate is a natural extract into which chromophore molecules have been coupled. A certain variability related to differences in the structure characterizes substrate batches (degree of polymerization, branching, etc.) and it is thus difficult to assert that the batches will contain the same level of chromophore. Therefore, it is necessary to define a reference scale with regard to a known enzyme activity each time a new batch of substrate is used.

These analysis methods, using a defined substrate and specific of an enzyme activity, are not affected by environmental interferences related to the reducing products and the interfering enzyme activities.

Methods that measure the reduction in viscosity of a solution or suspension of natural substrate are based upon the enzyme's ability to reduce the viscosity of a standard substrate solution under defined conditions of pH and temperature. The viscosity measurement is relative to the initial substrate viscosity. In this method, like the previous one, substrate quality is essential to the measurement accuracy and, in this case, the absolute viscosity of the batch of substrate (as affected by the degree of branching, etc.).

Although these methods give the best results in relation to applications in animal feed, the major inconvenience is the effort required to take a significant number of measurements. However, the methods may be automated.

As we have seen when analyzing enzyme in feed, viscometric methods and colorimetric methods with chromogenic substrates offer advantages over methods involving the release and measurement of reducing sugars. In the matters of implementation and costs, each of these methods present advantages and disadvantages; nevertheless, all of them are characterized by their dependence on the quality of the substrate.

Depending on the assay method used, a single enzyme preparation can give different numerical results (Tables 2 and 3). Clearly the number of units of enzyme activity obtained from different assay results have no bearing on enzyme performance in feed.

A criticism that can be made about these methods is that they do not make use of the substrate found in the feed; this is particularly the case for methods based on chromophore coupled substrates. Moreover, such methods, although founded on the same principle, may be implemented differently (in terms of method of enzyme extraction, buffer, pH, temperature) because of the origin of the enzymes.

The analytical conditions recommended by the enzyme manufacturers for the measurement of xylanases in feed are summarized in Table 4. As shown, the methods can be significantly different in terms of buffer, pH, and the assay duration. Does this reflect so-called "methodological problems?"
TABLE 2. Endo-1,3(4)-β-glucanase assayed by different analytical methods

<table>
<thead>
<tr>
<th>ORIGIN OF ENZYME</th>
<th>A</th>
<th>B</th>
<th>C</th>
</tr>
</thead>
<tbody>
<tr>
<td>Endo-1,3(4)-β-glucanase from Aspergillus niger</td>
<td>1</td>
<td>2.9</td>
<td>6.7</td>
</tr>
<tr>
<td>Endo-1,3(4)-β-glucanase from Penicillium funiculosum</td>
<td>1</td>
<td>1.8</td>
<td>3.6</td>
</tr>
<tr>
<td>Endo-1,3(4)-β-glucanase from Geosmithia emersonii</td>
<td>1</td>
<td>8.5</td>
<td>11.1</td>
</tr>
</tbody>
</table>

*A* = Endo-1,3(4)-β-glucanase: azo-barley β-glucan substrate, chromophore release measurement; *B* = Endo-1,3(4)-β-glucanase: barley β-glucan substrate, reducing sugar (DNS) measurement; *C* = Endo-1,3(4)-β-glucanase: barley β-glucan substrate, change in relative viscosity measurement.

*B* Activities are normalized to method A, measured with the chromophore substrate.

TABLE 3. Endo-1,4-β-xylanase assayed by different analytical methods

<table>
<thead>
<tr>
<th>ORIGIN OF ENZYME</th>
<th>A</th>
<th>B</th>
<th>C</th>
</tr>
</thead>
<tbody>
<tr>
<td>Endo-1,4-β-xylanase from Trichoderma longibrachiatum</td>
<td>1</td>
<td>6.7</td>
<td>8.0</td>
</tr>
<tr>
<td>Endo-1,4-β-xylanase from Geosmithia emersonii</td>
<td>1</td>
<td>17.3</td>
<td>8.8</td>
</tr>
</tbody>
</table>

*A* = Endo-1,4-β-xylanase: azo-oat xylan substrate, chromophore release measurement; *B* = Endo-1,4-β-xylanase: birchwood xylan substrate, reducing sugar (DNS) measurement; *C* = Endo-1,4-β-xylanase: wheat arabinoxylan substrate, change in relative viscosity measurement.

*B* Activities are normalized to method A, measured with the chromophore substrate.

TABLE 4. Analytical conditions recommended for five different xylanase products

<table>
<thead>
<tr>
<th></th>
<th>XYLANASE A</th>
<th>XYLANASE B</th>
<th>XYLANASE C</th>
<th>XYLANASE D</th>
<th>XYLANASE E</th>
</tr>
</thead>
<tbody>
<tr>
<td>Buffer</td>
<td>Acetate</td>
<td>Citrate</td>
<td>Acetate</td>
<td>Acetate</td>
<td>Acetate</td>
</tr>
<tr>
<td>pH</td>
<td>4.7</td>
<td>4.8</td>
<td>5</td>
<td>6</td>
<td>4</td>
</tr>
<tr>
<td>Temperature</td>
<td>30°C</td>
<td>50°C</td>
<td>40°C</td>
<td>50°C</td>
<td>50°C</td>
</tr>
<tr>
<td>Incubation time</td>
<td>20 min</td>
<td>300 min</td>
<td>60 min</td>
<td>120 min</td>
<td>30 min</td>
</tr>
</tbody>
</table>

ARE THERE METHODOLOGICAL PROBLEMS IN MEASURING ENZYME ACTIVITY IN THE FEED?

We have previously demonstrated that the analysis methods are dependent on the product origin and that the different methods and substrates produces different results.

Any methodological problem is primarily concerned with establishing the correct assay procedures, as much a function of the experimental conditions as substrate quality. Enzyme manufacturers have developed analytical methods for each enzyme, as reliable methods are an absolute requirement for monitoring enzyme production and the specification of the final product.

However, it is not possible to define a universal and standard method for an enzyme activity, since enzymes, ostensibly with the same molecular action, cannot be assayed under the same conditions (e.g. pH and temperature). If one takes into account the specificity and characteristics of a particular enzyme, then for a given enzyme from a defined origin, one or more assay methods will give good results under the appropriate and defined conditions.

Analysis of enzyme activity in feed presents a methodological problem. The feed may contain not only endogenous enzyme activities that need to be accounted for, but also interfering factors as components of the feed. These components may be produced during formulation or result from the processing of feed during manufacture. Differences in feeds may be introduced during manufacture as a result of variations in formulation, quality of the components, and conditions of manufacture. Therefore, the risks of interferences with the enzyme measurement in feed have to be considered. Furthermore, cellulolytic activi-
ties have the ability to bind to feed components, so measuring in-feed activity with an inefficient extraction method may be difficult.

In Figure 3 the effect of the feed constituents on the xylanase activity has been summarized. These data were presented by B.V. McCleary during the "Second European Symposium on Feed Enzymes" (Noordwijkerhout, Netherlands, October 25-27, 1995). In this experiment, McCleary measured the activity of four xylanase dose rates from Trichoderma longibrachiatum introduced in the feed; then he drew an absorbance curve of the in-feed enzyme activity and an absorbance curve of the enzyme activity in the absence of feed. This study demonstrates that interference occurs between the feed and the measurement of the enzyme activity with chromogenic methods as well as with viscometric methods. Such interferences give variable results from one product to another. In order to get rid of this variability it is essential during each analysis to establish a product calibration curve using an internal standard.

It is clear that we can solve the methodological problems of the enzyme activity measurement in feed by adapting a specific analytical method to each case.

**FIGURE 3.** Xylanase activity from Trichoderma longibrachiatum in feed (comparison between the TRX activity + feed and the TRX activity in the absence of feed)

---

**CONCLUSIONS AND APPLICATIONS**

1. Using enzymes as feed additives requires us to address a number of concerns, the two most important of which are choosing a product and controlling its addition to the feed.

2. For each enzyme activity, defined by reference to the microorganism which produced it, there is a measurement method recommended by the supplier. On the other hand, when using such methods to measure enzyme in feed, it is necessary to take into account the specific nature of the feed and to adapt the method accordingly.

3. It is impossible to make an enzyme selection on the basis of the enzyme units only; they are not as significant as they are specific to the origin of the enzyme and to the analytical method.

4. Except for product stability, the only comparison of enzyme remains the *in vivo* assay. Let the animal be the judge!