Xylanase inhibitors in UK wheat varieties: Survey of incidence and significance to pig and poultry feed

by

T. Verhoeven[1], F. Short[1], R. Weightman[1],
A. Cowieson[2] and M. F. Isaksen[2],

[1]ADAS Consulting Ltd
Woodthorne, Wergs Road, Wolverhampton, WV6 8TQ

[2]Danisco Animal Nutrition Ltd, PO Box 3, Boothby Graffoe, Lincoln, Lincolnshire LN5 0XB

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Abbreviations used

AA	Amino Acid
AME	Apparent Metabolisable Energy
AMEn	Apparent Metabolisable Energy corrected to nitrogen balance
AMY	Amylase
AX	Arabinoxylan
BPC	British Poultry Council
BWG	Body Weight Gain
FCE	Feed Conversion Efficiency
FCR	Feed Conversion Ratio
G x E	Genotype x Environment
GH	Glycoside Hydrolase
GI	Gastro-Intestinal
ME	Metabolisable Energy
NSP	Non-starch polysaccharides
pRIXI	Putative Rice Xylanase Inhibitor
TAXI	*Triticum aestivum* Xylanase Inhibitor
TL-XI	Thaumatin-Like Xylanase Inhibitor
XI	Xylanase Inhibitor
XIP	Xylanase Inhibitor Protein

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Executive Summary

This review was undertaken to investigate the impact of xylanase inhibitors (XIs) in wheat on UK industries such as animal feed and enzyme manufacturers, poultry producers and wheat growers, and indicate how these sectors could benefit from further research and development in this area.

Reduction of viscosity in wheat-based animal feed

Wheat is a major ingredient in animal feed (typically up to 65 %) but non-starch polysaccharides (NSPs), and mainly the arabinoxylans (AX) in feed wheat confer high viscosity in the GI tract. For the past two decades, wheat-based feed for monogastric animals (pig and poultry) has been supplemented with enzyme cocktails (exogenous enzymes derived from fungal or bacterial sources) to reduce viscosity in digesta. AX hydrolysis by enzymes such as xylanases reduces viscosity and results in improved digestion, improved Feed Conversion Efficiency (FCE) and healthier flocks, particularly with young, growing birds such as broilers.

Presence of XIs in wheat

In recent years, several xylanase inhibitor (XI) proteins have been found in wheat and other cereals, and have been described in the scientific literature. Most studies have focussed on protein characterisation, their mode of action, substrate specificity, occurrence and distribution within the plant. Only few reports illustrate how XIs present in wheat may interfere with current industrial processes relying on xylanase addition for product improvement.

Variation in XIs between wheat varieties

XIs have been found in all wheat varieties tested so far. Most varieties tested were bread wheat varieties, although some feed wheat was also included. In addition, most work reported has been on white flour, whereas for feed purposes, characterisation should be carried out at the whole-grain level.

The levels of XIs in European wheat reported in the literature vary between wheat sources, and may be partly environment dependent. The impact of XIs may be greater on feed wheat varieties *vs.* bread wheat varieties: most feed wheats contain the 1BL/1RS translocation and are highly viscous, thus needing more exogenous xylanase addition to reduce viscosity. The continental work showed that the varieties Rialto and Cadenza, both 1BL/1RS wheats, contained high levels of XIs. In addition, all the non-1BL/1RS feed wheat varieties tested also tested high for XI levels. To specifically quantify how XIs affect wheat quality and exogenous enzyme performance in the UK, the correlation between wheat AME and XI levels needs to be investigated using UK-grown wheat varieties.

Interaction between XIs and commercial enzymes

Both poultry and non-poultry producers have been relying on addition of xylanase-containing enzyme cocktails to reduce feed viscosity in monogastric animals. However, enzyme efficacy is variable. There are several possible reasons for this, but the XIs present in wheat, which makes up the bulk of feed, are likely to contribute to this variability as it has been shown that the commercial enzymes are inhibited by XIs.

Survival of XI during the feed manufacturing process

XI survival during feed processing has not been tested, and such knowledge is lacking regarding how much actual inhibition is still present in processed feed. All previous research has focussed on wheat and/or wheat flour and has used purified proteins and inhibitors. The actual amount of XI present in the end product after processing is likely to differ significantly from the starting material, and other components present in processed feed may also impact on xylanase and XI interactions. Therefore, an interesting approach for the feed industry would be to study the effect of processing at different temperatures, water contents, pressures and recipe on the survival of the inhibitors, and the nutritional value of the feed in relation to actual XI activity.

Quantification of XI impact on xylanase performance in feeds

Currently, feed trials researching the benefits of xylanase addition do not take the presence of XIs into account, nor the fact that different feed wheat varieties may contain varying XI levels. Therefore, to address optimal quantification of xylanase performance in wheat-based animal feed, more research needs to be conducted. Such research will need to be carefully designed to ensure that results are not artefactual and that they reflect the action of the native (endogenous) XIs.

Screening requirements for XIs in UK wheats

Even though few of the varieties tested so far are currently grown in the UK, and few are representative of feed types, it is to be expected that similar levels of XIs are present in UK lines. However their impact has not been quantified.

Initial screening of XI levels in UK-grown feed wheat appears the simplest and most direct way to establish variation. This would also help direct future research, such as more extensive investigations into potential new low-XI wheat breeding lines, wheat nutritional quality and animal feed performance.

Development of new XI assays

All assays reported in the literature currently rely on spectrophotometric assays, and these are not representative of the environment in which the enzymes would normally work. A more realistic way of measuring XI impact on feed quality would be through quantifying their impact on viscosity, with and without exogenous xylanases. Enzyme companies currently use similar methods to quantify the impact of exogenous xylanases on feed viscosity. In addition, assays described thus far have not accounted for possible xylanase contamination from fungal residues potentially present on grains, thereby possibly underestimating xylanase inhibition. It is not clear to what extent previous studies have been influenced by endogenous levels of xylanases in the grain or contamination with enzymes of fungi colonising grain after harvest. This impact should be quantified.

Recommendations

A number of areas for further work and development were identified:

1. Assessment of varietal variation in XIs in UK wheat by rapid, informative lab-based screening;
2. Development of XI level assays adapted to the animal feed industry;
3. Evaluation of fungal xylanase contamination in stored feed wheat;
4. Studies on XI survival during feed processing;
5. Quantification of xylanase performance in wheat with and without XIs.

1. Introduction

Wheat is a major ingredient in poultry feed, typically comprising up to 65 % of the diet (R. Clark-Monks, BPC, *pers. comm.*). Annually, 6.3 million tonnes of wheat is used in poultry feed production in the UK (Defra 2005). Variability of performance of UK wheat in monogastric diets is an ongoing concern, and this review investigates one aspect of this variability relating to enzyme technology and the presence of enzyme (specifically xylanase) inhibitors in wheat. The aim of the study was to enable the UK industry to keep abreast of research that has been carried out elsewhere in Europe, and to indicate avenues for further research where appropriate in a UK context.

1.1. Historical context

In the late 1980's, problems with pig and poultry diets containing high levels of wheat became apparent. In some instances, animal performance deteriorated, and this was associated with problems such as sticky droppings and hock burn in broilers, and colitis in pigs. At the time, it was postulated that this was related to the introduction of a new wheat type containing the 1BL/1RS translocation from rye, which had higher disease resistance. A typical 1BL/1RS feed variety introduced at that time was Slejpner, which was also reported to contain higher levels of non-starch polysaccharides (NSP). However, the exact cause of these digestive problems was not clear: other traits such as more vitreous grains and differences in protein quality were also a characteristic of 1BL/1RS-wheats. The problem was worse in young animals with a high feed intake and poorly developed digestive systems.

One of the features of rye, and to a lesser extent of 1BL/1RS-wheats, was (and still is) an increased viscosity when flour is slurried with water. It was postulated that the high viscosity was principally due to the presence of 'pentosans' (now more commonly termed arabinoxylans; AX) in the NSP fraction, which are indigestible to the monogastric animal, but can be fermented in the large intestine. At its simplest level, ingestion of feed of potentially high viscosity meant that the animal would consume more water and less feed and so performance would decline. Moreover, a rapid fermentation in the large bowel and release of water bound by the NSP could lead to diarrhoea and associated health problems. Reducing viscosity in order to improve animal performance became a key goal.

At around the same time, feed enzymes were introduced by companies such as Finfeeds, Danisco and Novo Nordisk. These exogenous enzymes could be added to the feed (either as a powder prior to mixing, or sprayed in liquid form on to the outside of finished pellets), and results showed

significant improvements in feed performance. Research showed that these enzyme cocktails, which were rich in xylanase activity, quite effectively decreased the viscosity of digesta (Bedford and Classon, 1992) and in parallel improved feed performance. The hypothesis linking xylanase activity with hydrolysis of pentosans, reduction in digesta viscosity and improved animal performance became firmly established. Quickly, these enzymes were taken up by the industry and by the end of the 1990's, the animal feed market had become a significant user of enzymes in Europe, closely behind those supplying the food & beverage and cleaning market (Hayes 2005).

1.2. Current state of the art

Endo-β-1-4-xylanases and β-1-3, 1-4-glucanases have now been used commercially in diets that are based on wheat and barley for approximately 20 years with a return on investment such that in many of these 'viscous grain' markets, penetration is in excess of 90% for broilers (Bedford 2000). Furthermore, the mode of action of these enzymes has been intensively studied and as a result is now fairly well elucidated (Bedford 2000). There are two main modes of action that have been proposed for xylanase and β-glucanase in wheat- or barley-based diets. These include the viscosity theory and the so-called 'cage' effect (Bedford 2002).

The viscosity theory suggests that, as the incremental performance enhancement associated with the addition of these enzymes to poultry diets is beyond that which can be explained by the nutritive value of the released sugars, there must be an additional advantage conferred by the enzymes to the digestibility of the other nutrients in the diet. As high molecular weight soluble arabinoxylans and mixed-linked β-glucans have a high affinity for water, they have the ability to stimulate a geometric increase in the viscosity of the contents within the GI tract (Bedford and Classen 1992; Nilsson et al. 2000). High intestinal viscosity is negatively correlated with animal performance and nutrient digestibility, and is also associated with detrimental changes in the microbial flora within the distal GI tract.   Thus, facilitating a reduction in viscosity with exogenous enzymes confers a nutritional advantage to the animal (Adeola and Bedford 2005; Bedford and Classen 1992; Cowieson et al. 2005; Meng et al. 2005; Sieo et al. 2005; Van der Kils et al. 1993).

The 'cage' effect theory is associated with the effects of carbohydrases on cell walls, reducing their integrity and thus releasing nutrients that were previously encapsulated (Bedford, 2002). It is likely that both mechanisms are involved in the responses to xylanase and β-glucanase in poultry diets. This would lead to beneficial changes in performance, nutrient retention, a reduction in incidences of sticky droppings and wet litter and desirable changes in the microbial flora in the distal GI tract (Bedford 2000; Choct et al. 2004; Graham et al. 2003; Shakouri and Kermanshahi 2005).

1.3. Outline of the present study

Despite the wide acceptance of the use of enzyme technology, there are still many questions surrounding the uses of xylanases in cereal-based diets. Enzyme performance is variable in commercial practice, but it is not known whether this is due to variability in enzyme quality, wheat quality, the manufacturing process or a combination of all of these.

Recently, some cereals including wheat have been found to contain endogenous xylanase inhibitors (XIs), which may reduce the activity of the exogenous enzymes added to wheat-based feeds. Since *in vitro* experiments have shown that xylanase inhibitors interact with xylanases (e.g. Bonnin et al., 2005 and Gebruers et al., 2002), it is possible that digestibility and energy utilisation could further be improved by understanding the interactions between XIs and xylanases. This could result in increased productivity in the poultry sector, resulting in greater demand for feed wheat, and/or reduce some of the variability in feed performance seen in commercial practice.

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This project was commissioned by the Home Grown Cereals Authority to review the information currently available on XIs. Chapter 2 reviews the published literature on XIs in wheat, summarises the work carried out in Europe, and discusses this in the context of wheat varieties available for growing in the UK. Chapter 3 discusses the likely effects of XIs on commercial enzyme performance. Chapters 4 and 5 attempt to quantify the impact of XIs on poultry feed performance and the likely costs of screening for them, and Chapter 6 summarises future research requirements.

2. Literature review: Xylanase inhibitors in wheat for feed applications

2.1. Non-starch polysaccharides in wheat-based feeds

Arabinoxylans (AX) and β-glucans are the major constituents of a group of cereal biopolymers known as non-starch polysaccharides (NSP). They make up the bulk of the cell walls, and are present in sufficient amounts to affect cereal processing and/or quality of the end product, AX being more important in wheat and rye, and β-glucans in oats and barley. Based on their solubility in water, water-soluble and water-insoluble AX can be distinguished.

In wheat, the amount and kind of AX present in the grain influences its use in several biotechnological applications, e.g. in milling, bread making, gluten-starch separation, distilling for potable alcohol and bioethanol, malting and processing of animal feed. For example, in feed most of the problems are caused by water-soluble AX and are closely linked with increases in digesta viscosity. As discussed in Section 1.2, increased viscosity of the feed ultimately results in decreased nutrient absorption, which accounts for reduced growth (Scott 2003). Cereal grains are added to poultry diets primarily as a source of energy because the protein content of most cereal grains is low (< 150g/kg) and has a poor amino acid (AA) profile, being particularly deficient in the sulphur amino acids and lysine. Thus, wheat 'quality' may be essentially defined as the relative content of 'apparent metabolisable energy' (AME). AME is expressed in MJ/kg and is a function of the content and digestibility of the protein, starch, oil and NSP in the grain, as well as the presence of anti-nutritional factors that may limit digestibility or increase endogenous energy loss.

2.2. The mechanism of action of xylanases in poultry and pig feed

Commercial enzyme preparations contain a 'cocktail' of different enzyme activities. Several different enzymes can hydrolyse AX, each hydrolysing specific bonds within the AX chain. Examples of xylan-hydrolysing enzymes and their modes of action are shown in Figure 1. Endoxylanases (E.C. 3.2.1.8[1], further referred to as xylanases) hydrolyse the glycosidic linkages in the main xylan chain, releasing xylo-oligosaccharides (Figure 1). Most of the xylanases belong to one of two distinct families, glycoside hydrolase (GH) family 10 or 11:

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[1] E.C. numbers (Enzyme Commission numbers) are a numerical classification scheme for enzymes, based on the chemical reactions they catalyse. As a system of enzyme nomenclature, every EC number is associated with a recommended name for the respective enzyme.

- GH 10 contains plant, fungal and bacterial xylanases;
- GH 11 contains fungal and bacterial xylanases structurally unrelated to GH 10 (Coutinho and Henrissat 1999).

Family 10 xylanases have higher molecular weights than family 11, are more complex and produce smaller oligosaccharides. Family 11 xylanases are more specific for xylan than family 10 (Jeffries 1996). Several xylanase preparations from microbial origin are commercially available as additives to wheat-based poultry and pig feed, in order to degrade or modify the xylan content of the feed.

Because there is a logarithmic relationship between molecular size of the AX and its viscosity in solution (and xylans are essentially long, linear chains), xylanase enzymes can effect rapid reductions in viscosity through a relatively small number of incisions in the main xylan backbone. This makes these enzymes very effective when used at low rates when applied to feed.

Further in this review, another two non-xylanase GH families will be mentioned, GH13 and GH18:
- GH13 contains polysaccharide-degrading enzymes, such as α-amylases;
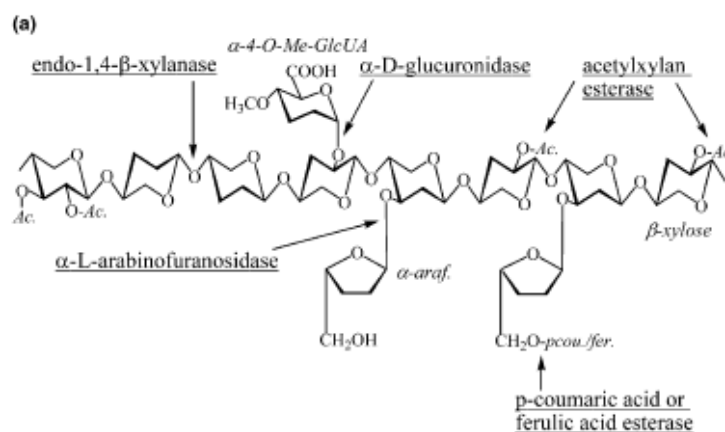- GH18 contains mostly chitinases.

Figure 1.
Structure of xylan and the sites of its attack by xylanolytic enzymes. The backbone of the substrate is composed of 1,4-β-linked xylose residues. Ac., Acetyl group; α-araf., α-arabinofuranose; α-4-*O*-Me-GlcUA, α-methylgucoronic acid; pcou., *p*-coumaric acid; fer., ferulic acid (Collins et al. 2005).

2.3. The potential impact of xylanases in poultry and pig feed

The quantitative effect of addition of exogenous xylanases to poultry feed depends on the AME of the feed wheat, and soluble NSP (a factor in feed viscosity) is responsible for much of the variation in AME. Therefore, feed containing wheat with low AME due to high viscosity will show a better response to xylanase addition than feed containing wheat with high AME (due to lower viscosity). For example, addition of exogenous xylanases to feed wheat with relatively low AME (e.g. 11MJ/kg) may improve AME by 0.5 – 1MJ/kg, whereas improvements in the range of 0.1 – 0.5MJ/kg would be expected in wheat with higher AME (e.g. 14MJ/kg). Even though there may be other factors explaining variance in response to xylanase in feed (diet design, nutrient balance, other ingredients, starch digestion, etc.), wheat viscosity (and thus AME) is a major factor. Increasing AME of the wheat added to poultry feed will improve the overall Feed Conversion Efficiency (FCE) of the feed assuming that a correct balance between AME and available amino acids can be maintained.

2.4. Xylanase inhibitors in wheat

Even though the addition of xylanases or enzyme cocktails containing xylanases have improved energy intake and FCE for broilers fed on wheat-based diets, significant variability in animal performance still exists. This is not surprising, since a great deal of variation is due to variability between animals. Also, there may be variation in the kind of wheat used for the feed (wheat cultivars, variation in AME within a variety), feed design and preparation (e.g. ME:AA ratio, conditioning temperature), administration and additives used. Variation due to product inhibition and differences in enzyme production batches is theoretically also possible.

Some known chemical xylanase inhibitors such as metal ions, SDS, *N*-bromosuccinimide and iodo acetic acid (Goesaert et al. 2004) also exist, but are unlikely to occur in animal feed. However, it has become clear that another component, inherently present in wheat, may be responsible for some of the variation in xylanase performance in cereal based diets. The discovery of a new type of enzyme inhibitor suppressing xylanase activity has been reported in wheat and other cereals (Debyser et al. 1997; Elliot et al. 2003; Goesaert et al. 2003a; Goesaert et al. 2003b; McLauchlan et al. 1999). Such xylanase inhibitor (XI) activity may be responsible for some of the variation in xylanase performance. So far, three classes of XIs have been identified in wheat: *Triticum aestivum* Xylanase Inhibitor (TAXI-) type inhibitors (Debyser et al. 1999; Gebruers et al. 2001), Xylanase Inhibitor Protein (XIP-) type inhibitors (Elliot et al. 2003; Juge et al. 2004; McLauchlan et al. 1999), and a Thaumatin-Like Xylanase Inhibitor (TL-XI) (Gebruers, Fierens et al. 2004). These are discussed in more detail in sections 2.4.2 to 2.4.4.

2.4.1. Xylanase inhibitor mode of action

Of the three types of inhibitors discussed, both the TAXI- and XIP-types use competitive inhibition as a mode of action (Gebruers, Brijs et al. 2004; McLauchlan et al. 1999), see also Figure 2. The strength of inhibition can vary depending on the specific xylanase and inhibitor, the concentration of each of them, the concentration of xylan and physicochemical factors such as pH and temperature, but the mode of action remains the same.

Theoretically XIs can be saturated with substrate (in this case the xylanase enzyme) and further added xylanases would no longer be inhibited. However, concentration of inhibitors far exceed the concentration of exogenous enzyme added to the feed. Using the figures reported in Bonnin et al. (2005), the concentrations of XIP and TAXI (moles) exceed the concentration of exogenous xylanases (moles) in most feed by between 100-700 times, depending on the type and concentration of inhibitor and enzyme. The reaction between moles of inhibitor and moles of xylanase is 1:1.

Arabinoxylan

Xylanase inhibitor

Xylanase

1) No inhibitors present

Xylanase binds to xylan and hydrolyses glycoside linkage

2) XI present

XI competes with xylan for xylanase active site

If xylanase binds XI, a complex is formed

If xylanase binds to xylan, product is still formed

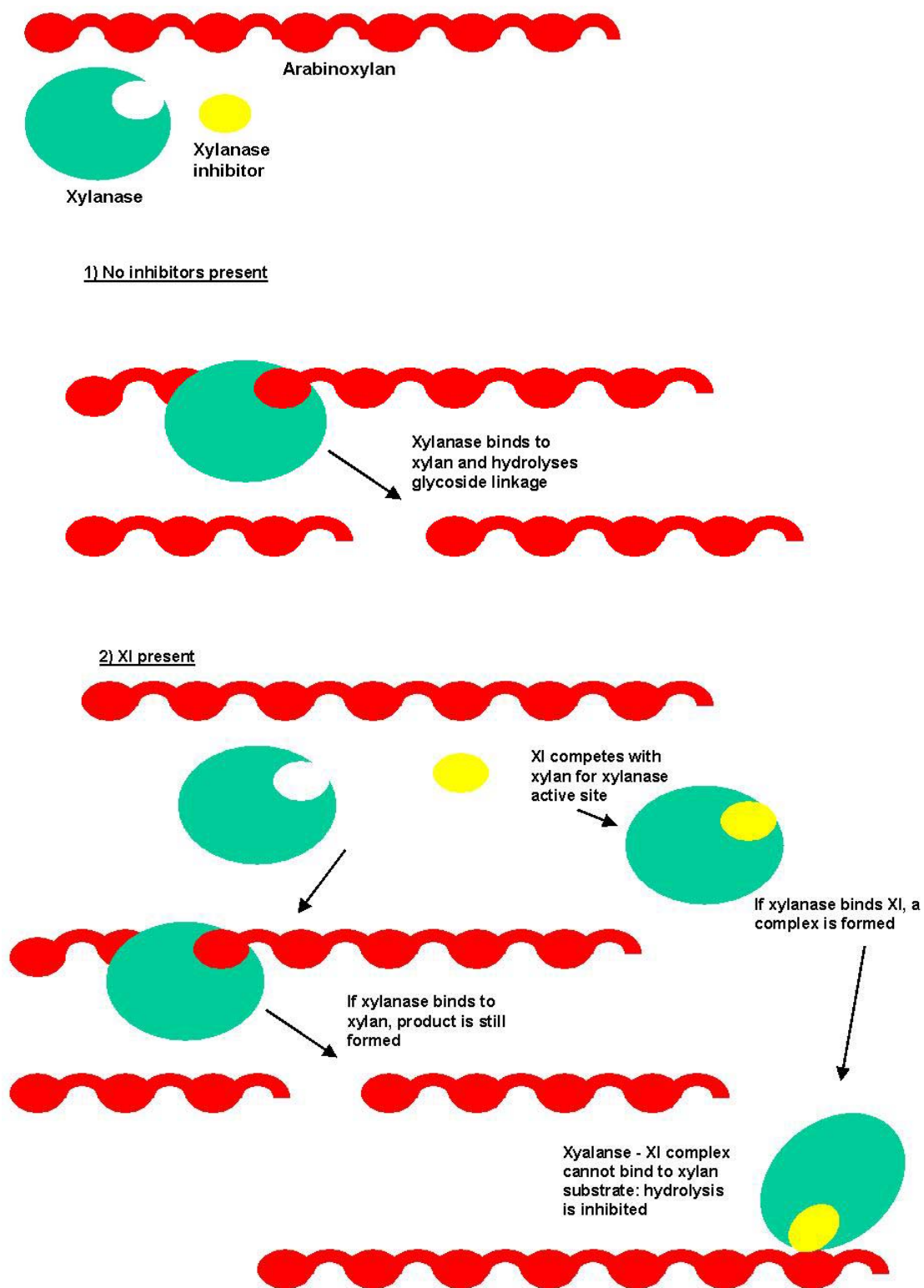Xyalanse - XI complex cannot bind to xylan substrate: hydrolysis is inhibited

Figure 2. Competitive xylanase inhibition

2.4.2. TAXI-type xylanase inhibitors

The TAXI protein (Debyser et al. 1997; Debyser et al. 1999); Eur. Pat. WO98/49278) was discovered when it was found to inhibit the barley malt xylanolytic system during mashing. Since then, the TAXI protein has been characterised further and several homologues have been found in other cereals, such as durum wheat, barley and rye.

Potentially four TAXI-type inhibitors have been discovered in wheat, but so far only the presence of two of them has been confirmed in flour. TAXI-type XI extracted from wheat flour consists of a mixture of two proteins, TAXI-I and TAXI-II (Gebruers et al. 2001). Both inhibitors have been purified and, even though they share many similarities, have been shown to exhibit different activities and inhibit different xylanase classes (see section 2.2). They have similar N-terminal amino acid sequences, have molecular masses of approx. 40kDa and are basic proteins (pI for TAXI-I is approx. 8.8; pI for TAXI-II is 9.3 or slightly higher).

Both proteins occur in two different molecular forms, form A (40kDa, one fragment) and form B (two fragments, 10 and 30kDa) (Gebruers et al. 2001). It is thought that the polypeptides that constitute form B are derived from A through modification. It is still unknown whether this modification is necessary to either activate form B or make it more active than form A. However, the authors argue that since both forms are present in wheat flour and no proteolytic modification occurred during their purification, it is also possible that both forms are present and active in wheat / wheat flour (Gebruers et al. 2001). More research would be required to verify this.

Recently, two more TAXI-type inhibitors have been found in wheat (Igawa et al. 2004). Unlike TAXI-I and II, they have not yet been purified but two mRNA species, *Taxi-III* and *Taxi-IV*[2], have been identified in wheat. At the nucleotide sequence level, *Taxi-III* and *Taxi-IV* are 91.7% and 92.0% identical, respectively, to *Taxi-I*, and *Taxi-III* and *Taxi-IV* are 96.8% identical. Transcripts of *Taxi-III* and *Taxi-IV* accumulate in roots and older leaves of wheat plants, and these increase significantly when plants are infected with *Fusarium graminearum* and *Erysiphe graminis* (powdery mildew), two fungal pathogens known to attack wheat. In contrast, transcript levels of *Taxi-I* do not increase significantly upon attack from these pathogens. Recombinant TAXI-III protein was shown to inhibit several purified xylanases from both fungal and bacterial origin.

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[2] Genes and loci are referred to with the first letter capitalised and the whole word italicised, e.g. *Abc1*. When referring to proteins and antisera, the whole word is capitalised, e.g. ABC1. When referring to cDNA derived from the gene the word is not italicised, e.g. Abc1. **Beavis W** (1995). A standard for maize genetic nomenclature. *Maize Genetic Cooperation Newsletter*, **69**:182-184.

These results suggest that different homologues of TAXI-type inhibitors may have different roles, some in plant defence against pathogen attack. TAXI-I and II are found in all parts of the wheat plant (roots, shoots and leaves) and throughout development, as well as in developing and germinating grain (Elliot et al. 2003). Whether TAXI-III and IV proteins have the same distribution pattern, and more importantly, are present in wheat endosperm, remains unknown.

2.4.2.1. Inhibition specificity of TAXI-I and TAXI-II

Even though TAXI-I and II show high similarities in protein sequence, glycosylation, size and pI, in some cases they show varying levels of inhibitions towards xylanases from different sources (see Table 1). In general, TAXI-type inhibitors inhibit bacterial and fungal xylanases from GH family 11 only (Gebruers et al. 2001), but show no inhibition towards endogenous wheat xylanases (Elliot et al. 2003).

2.4.3. XIP-type xylanase inhibitors

Another type of xylanase inhibitor protein (XIP), has been found in wheat endosperm (Juge et al. 2004; McLauchlan et al. 1999). This protein is glycosylated, monomeric, with a molecular mass of approx. 30kDa and a pI of approx. 8.8. XIP-I belongs to GH family 18. GH18 contains mostly chitinases and other proteins grouped by sequence similarity. Chitinases catalyse the hydrolysis of β-1,4-N-acetyl-D-glucosamine linkages in chitin polymers. A conserved glutamate at the end of the active site signature is thought to be involved in the catalytic mechamism, and probably involved as a proton donor (Coutinho and Henrissat 1999; Watanabe et al. 1993). XIP-I shows high homology with plant chitinases but lacks enzyme activity. Even though the conserved glutamate is present, clear structural differences around the active site account for its lack of chitinase activity (Juge et al, 2004). It does however have xylanase inhibition activity, the specificity of which will be discussed further. It is now thought that the GH18 family contains several "inactivated chitinases" that inhibit xylanase activity competitively (Durand et al. 2005).

Several XIP-I isoforms (28.5, 29, 30kDa) have been extracted from wheat flour using antibodies (Elliot et al. 2003), and the highest yield of XIP-I extracted from 1kg of wheat whole meal extracts was around 20mg. XIP-type inhibitors have also been purified from durum wheat, barley, rye and maize.

2.4.3.1. Inhibition specificity of XIP-I

XIP-I has two independent binding sites, and acts through competitive inhibition, with Km_i values ranging from 3.4 to 610nM (Juge et al. 2004; McLauchlan et al. 1999). Due to the two binding sites present in XIP-I, it can recognise two different three-dimensional structures and distinguish

between fungal and bacterial xylanases from the same structural family. XIP-I is specific for fungal xylanases from GH families 10 and 11 (except for *A. aculeatus* GH10 xylanase, GENSEQP entry AAE69552), but does not inhibit bacterial xylanases, as shown in Table 1. XIP-I does not inhibit endogenous wheat xylanases. However, it was found to also inhibit 2 barley α-amylases (AMY1 and AMY2) of GH family 13. GH 13 is a large family containing polysaccharide degrading enzymes, e.g. amylase, isoamylase and pullulanase, grouped together based on sequence similarities (Coutinho and Henrissat 1999). The fact that one of the XIs described in detail so far inhibits other glycoside hydrolysing enzymes indicates XIs may also reduce starch digestibility in the animal.

2.4.4. TL-XI –type xylanase inhibitors

This last type of inhibitor, showing similarities to thaumatin, has only recently been discovered and not much is known about it. Thaumatin, a protein present in fruits of *Thaumatococcus daniellii*, is one of the most potent sweeteners known on a molar basis. It shares AA sequence similarity with some pathogenesis-related proteins, and thaumatin-like proteins have been implicated in pathogen defence. However, they may also play a role in flower development and fruit ripening (Arro et al. 1997). Interestingly, they have been shown to bind water-insoluble-1,3 glucans (Trudel et al. 1998), which may be how TL-XI inhibits xylan degradation by xylanases. TL-XI is glycosylated, basic (pI ~ 9.3) with a molecular mass of approx. 18 kDa. It has been isolated from wheat, and occurs as multiple isoforms. A number of GH11 xylanases are inhibited by this protein, but it is inactive towards GH10 xylanases (Gebruers, Fierens et al. 2004). Since more information on TL-XI is not yet available, all further references to XIs in this review will be based on the TAXI and XIP-type inhibitors.

2.4.5. Other xylanase binding proteins

Finally, a xylanase binding protein similar to rice chitinase has been found in rice (Goesaert 2002), as well as a putative rice xylanase inhibitor (pRIXI; (Durand et al. 2005). Even though the xylanase binding protein shows no xylanase inhibition activity, the fact that several xylanase binding and/or inhibitor proteins have been found in cereal grains shows that further presence of other xylanase interacting proteins cannot be excluded.

Table 1. Xylanase inhibition by wheat TAXI-type and XIP-type inhibitors (taken from Goesaert et al. (2004), with additional data).

| Endoxylanase | | Inhibitor | | |
Source / organism	Family	TAXI-I	TAXI-II	XIP-I
Fungal				
Aspergillus niger (Xyn1)	11	Yes	No	Yes
Trichoderma longibraciatum (M2)	11	Yes	Weak	Yes
Trichoderma longibraciatum (M3)	11	Yes	Yes	Yes
XBSni ^		No	No	●
Trichoderma viride (M1)	11	Yes	Yes	Yes
Penicillium funiculosum (XynC)	11	Yes	Yes	Yes
Penicillium funiculosum (XynB)*	11	Yes	No	Yes
Penicillium purpurogenum (Xyn B)	11	Yes	Weak	●
Penicillium purpurogenum (Xyn A)	10	No	No	●
Aspergillus nidulans	10	No	No	Yes
Aspergillus niger	10	No	No	Yes
Aspergillus oryzae	10	No	No	Yes
Aspergillus aculeatus	10	No	No	No[3]
Talaromyces emersonii	10	No	No	No
Bacterial				
Bacillus subtilis (XynA)	11	Yes	Yes	No
Bacillus agaradhaerens	11	●	●	No
Fibrobacter succinogenes (A)	11	●	●	No
Fibrobacter succinogenes (B)	11	●	●	No
Pseudomonas fuorescens	10	●	●	No
Cellvibrio japonicus	10	●	●	No
Bacillus sp.	10	●	●	No

^ Mutated *B. subtilis* xylanase (Frederix et al. 2004)

*Recombinant xylanase from *P. funiculosum* expressed in *Pichia pastoris* (Brutus et al. 2004).

●No testing reported

2.4.6. Temperature stability of xylanases inhibitor proteins

Both XIP and TAXI are inactivated by boiling (Elliot et al. 2003; Gebruers et al. 2001), and it is likely that TL-XI is too. TAXI-type xylanase inhibitors are stable at temperatures up to 70°C for 40min and for 2h at pH 3.0 – 12.0. Boiling the inhibitor for up to 4.5min will reduce activity by 50% (Gebruers, Brijs et al. 2004). To completely inactivate TAXI-I and II, these enzymes need to be boiled for 15 minutes or autoclaved (Gebruers et al. 2001; Sørensen et al. 2004). These results show that the TAXI-type inhibitors are fairly stable and unlikely to be inactivated through the feed

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[3] A. aculeatus XylAA was reportedly inhibited by XIP-I (Bonnin et al. 2005) but Gebruers et al. (2004) reported no inhibition by this enzyme. This suggests that more than one xylanase can be purified from A. aculeatus, and their interactions with XIP-I should be investigated in more detail.

manufacturing processes. Moreover, in the lower water content environment of an animal feed mix, the risk of degradation is further reduced.

2.5. Xylanase inhibition levels in different wheat varieties

TAXI-type and XIP-type XIs were originally isolated from *T. aestivum* L., var. Soissons, and most research on protein structure, biochemistry and substrate specificity has focussed on the inhibitors extracted from this bread wheat variety. However, there are a few publications that report the differences in xylanase inhibition between different wheat cultivars and in different parts of the same plant (Bonnin et al. 2005; Gebruers et al. 2002; Rouau and Surget 2002). In all these publications, variability in xylanase inhibition depending on wheat variety has been reported, even though the degree of variation differs slightly between reports.

2.5.1. Variation between varieties and between grain components

Rouau and Surget (2002) tested three wheat varieties, Apollo, Soissons and Thésée, for xylanase inhibition. They found variability in inhibitory effects between varieties as well as between different grain fractions within varieties. In this investigation, most of the inhibition was found in the endosperm but the bran fraction also accounted for some of the xylanase inhibition. Xylanases from different microbial origins as well as two commercially available xylanase preparations were tested, and all were strongly inhibited by the XIs present in all varieties tested. One commercially available arabinofuranosidase (Grindamyl[TM], Danisco, Denmark) was also found to be inhibited but to a lesser extent than the xylanases tested.

Gebruers et al (2002) also found that the bran fractions contained approximately 21% of the total activity, whereas the endosperm (flour) fractions accounted for 57%. More recently, Bonnin et al. (2005) reported that the average amounts of XIP calculated from 20 different wheat varieties were 0.32mg/g in flour and 0.41mg/g in whole grain, and for TAXI these values were 0.11mg/g in flour and 0.14mg/g in whole grain. It should be noted that the amounts of XIs present in flour (mainly endosperm) or whole grain (endosperm, embryo and bran) may differ within varieties tested. The distribution of XIP- and TAXI-type inhibitors in the grain may differ per variety, e.g. with some varieties containing more XIs in endosperm than in bran.

Therefore, it is important to consider that when whole grain is used in animal feed, it is likely to contain more XIs per g weight, than white flour, yet much of the European research has focussed on studies of white flour, in a breadmaking context.

2.5.2. Variation in xylanase inhibitor activity across a wide range of European wheats

Gebruers et al (2002) tested 19 different European wheat varieties for their quantitative and qualitative variation in inhibition activity against GH 11 xylanases of *A. niger*, *B. subtilis*, and *T. viride* and a GH 10 endoxylanase of *A. aculeatus*. Similarly, Bonnin et al. (2005) tested 20 French wheat cultivars on variation of XIs (both TAXI and XIP). Interestingly, the research conducted by both groups highlighted the fact that *A. aculeatus* may produce two different xylanases, one that is inhibited by XIP-I and one that is not (Bonnin et al. 2005). Alternatively, whole wheat may contain an as yet unidentified component other than XIP-I that also inhibits xylanase activity from *A. aculeatus*. For all the other xylanases tested, both groups reported significant variations in inhibitor levels in all varieties tested. A comparison between relative XI activities in the varieties tested by both groups is represented in Figure 3.

Table (Based on Bonnin et al., 2005 and Schlegel, 2005)

Xylanase inhibition	Thésée	Texel	Alexandre	Virtuose	Soissons	Bonpain	Malacca	Elephant	Sideral	Aligre	Albéric	Supersoft	Baltimore	Magdalena	Tamaro	Crousty	Apache	Isengrain	Sisley	Meunier
	Low			Medium								High								
UK RL 2005					✓		✓													
Previous UK RL					✓		✓													
1BL/1RS translocation																				

Based on Bonnin et al., 2005 and Schlegel, 2005

Xylanase inhibition	Cezanne	Baldus	Cyrano	Record	Soissons	Anemos	Poseidon	Bercy	Residence	Harrier	Semper	Vivant	Tremie	Reaper	Rialto	Farandole	Versailles	Ritmo	Cadenza
	Low				Medium						High								
UK RL 2005					✓														
Previous UK RL	✓				✓									✓	✓				✓
1BL/1RS translocation															✓				✓

Based on Gebruers et al., 2002 and Schlegel, 2005

UK RL: HGCA recommended list for UK-grown wheat

Figure 3. Comparison of relative xylanase inhibition in different wheat varieties.

Very few of the wheat varieties tested are currently grown in the UK, and most of them are varieties used for bread making rather than animal feed. The varieties Crousty, Elephant, Harrier, Reaper, Tremie and Vivant are known feed wheats (D. Thompson, Syngenta Seeds Ltd., *pers. comm.*), and all have high levels of XIs apart from Elephant and Harrier, which have a medium level (Figure 3).

Only two of the varieties tested are known to have the 1BL/1RS translocation, which may also influence viscosity. This will be discussed in more detail in section 2.6.

Gebruers et al. (2002) found that the inhibitor levels (i.e. the quantity of inhibitor needed to decrease activity of 1 unit of xylanase by 50%) in different fractions of different European wheat varieties varied with a factor of 2-3, and that inhibitor activities are linearly related. This means that either the levels of different XIs with different substrate specificities are linearly related, or that one (or more) of the inhibitors are causing almost all of the inhibition activity measured.

2.5.3. Potential impact of G x E interaction on xylanase inhibitor activity

One other important observation is that significant variation of XI activity can exist within one wheat variety (Gebruers et al. 2002). The authors suggest this could be attributed to differences in environmental conditions (climate, soil), time of harvest and/or storage conditions. It should be noted that the wheat varieties tested by Gebruers et al. (2002) were commercially obtained and it is therefore likely that they were not grown in the same location. The effect of different growing, harvesting and storage conditions on the XI levels in different wheat varieties requires further investigation, as this was not addressed in their study. The variation of xylanase inhibition within one of the cultivars tested (Cadenza) was within the range of the variation reported between cultivars, but the values were not extreme.

Even though there may be relationship between environmental conditions and XI levels in wheat, the results reported by Bonnin et al. (2005) are important because all the varieties tested had been grown in one location, and significant variation between xylanase inhibition in different wheat varieties was shown. Unfortunately, comparison of xylanase inhibition reported by different research groups is difficult, as different extraction methods, assay methods and units of measurement to express inhibition have been used. This will be further discussed in section 4.1.

2.6. The impact of xylanase inhibitors in feed wheat varieties currently grown in the UK

As discussed, most of the research on XIs in cereals has been conducted outside the UK. Therefore, only a few of the varieties tested are currently grown in the UK and most of these are for milling and bread making, e.g Soissons. However, the Pedigree of these cultivars can be assessed and the likelihood of a link with UK feed wheat varieties can be examined.

Feed viscosity is one of the important quality characteristics required in feed wheat. Many of the UK feed wheats contain the 1BL/1RS translocation from rye (Schlegel 2005), and a correlation has been found between the presence of 1BL/1RS and high viscosity (Weightman et al. 2001). Most of the varieties tested previously (Bonnin et al. 2005; Gebruers et al. 2002) do not have the 1BL/1RS translocation, as most were high quality bread-making wheats. However, the two varieties that do (Cadenza and Rialto), also have some of the highest XI levels reported (Figure 3). The feed wheat varieties tested do not contain the 1BL/1RS translocation but still have high levels of XI activity. Therefore, it is important to establish whether UK feed wheats, many of which are also 1BL/1RS, similarly contain high XI levels. If so, the presence of XI would confer a greater disadvantage in feed wheat varieties than in bread making varieties, since 1BL/1RS wheats used for feed are mostly also high-viscosity wheats.

3. Impacts of xylanase inhibitors on feed performance

The previous section described the published data on XI activity in wheat, in relation to the more theoretical aspects of enzyme binding and protein structure. The following section discusses the potential impact of XIs from the perspective of the feed industry and enzymes suppliers.

3.1. Effects on enzyme performance in the commercial situation

The susceptibility of exogenous enzymes to inhibition is the subject of some discussion within the industry, as its relevance in wheat is not clear. However, some recent work by Ponte and co-workers concluded that the concentration of XIs in wheat was responsible for limiting the efficacy of exogenously added xylanase (Ponte et al. 2004). Ingelbrecht and colleagues also noted that the efficacy of exogenous xylanase was compromised by the presence of XIs related to rheological properties of doughs and that differences in the efficacy of different xylanases may be ascribed to differences in the degree of inhibition between xylanases (Ingelbrecht et al. 2000). Furthermore, there is considerable variation in the concentration of XIs in wheat (see previous Sections 2.5-2.6) all of which may contribute to variation in wheat AME and the range of responses that can be expected by using exogenous enzymes.

However, some researchers have concluded that most XIs use a competitive exclusion mechanism and so may become saturated with substrate (exogenous xylanase) quickly (Gebruers, Brijs et al. 2004; McLauchlan et al. 1999), thus leaving enough active xylanase to achieve the desired physiological effects. This may not be the case however: as described in section 2.4.1, there is approximately 250 times more inhibitor present in wheat than the amount of xylanase added. The reason complete inactivation of exogenous xylanase does not occur commercially may be related to the lack of contact between the two compounds, caused primarily by the low moisture content of feed.

Whether or not XIs have adverse effects on the efficacy of exogenous xylanase(s) *in vivo* is therefore not totally clear, especially in instances where the feed is conditioned at high temperatures although the evidence suggests that these proteins are thermostable to a degree (Gebruers et al., 2004; see Section 2.4.6).

3.2. Effects on enzyme recovery

Regardless of the true effects of XIs *in praxis*, there is a significant cost associated with the presence of these compounds in cereals for suppliers of feed enzymes; variation in the recovery of enzyme activity in feed has a negative effect on customer perception of product quality and

consistency. The above studies suggest that substrate affinity and the degree of inhibition should be factors that are considered in the development of the next generation of fibre-degrading enzymes for monogastric diets.

3.3. Efficacy of enzymes in non-broiler poultry species

There have been a number of recent papers published to assess the efficacy of NSP-degrading enzymes for other 'non-broiler' poultry species, probably stimulated to a degree by the requirements of the EU legislative bodies for registration of enzyme products. A table summarising some of the recent trials is presented below (Table 2). As in the case with poultry feed, the nutritional value of wheat-based diets could in most cases be improved by supplementation of the diet with exogenous xylanase. However, this was again dependent on parameters such as wheat viscosity, quality of the feed ingredients being offered (i.e. the inherent AME of the cereal, or more correctly, the inherent FCR of the cereal), bird age, animal environment and/or physical processing of the diets. This was true for ducks (Adeola and Bedford 2005), turkeys (Mathlouthi, Juin et al. 2003) and laying hens (Lazaro et al. 2003; Mathlouthi, Mohamed et al. 2003).

Table 2. Effect of supplementation of poultry diets with xylanase and/or β-glucanase on the performance of broilers, ducks, turkeys and laying hens.

Diet	Enzyme	Species	% improvement over control (parameter)	Reference
Wheat	Xylanase	Ducks	~9% (FCR); ~12% (BWG)	Adeola and Bedford, 2005
Wheat/Rye	Xylanase	Broilers	~6% (FCR); ~4% (BWG)	Cowieson et al., 2005
Wheat/Barley	Xylanase Glucanase	Turkeys	~2% (FCR)	Mathlouthi et al., 2003
Wheat/Barley	Xylanase Glucanase	Layers	~4% (FCR)	Mathlouthi, Mohamed et al. 2003
Maize	Xylanase Glucanase	Layers	~3% (FCR)	Mathlouthi, Mohamed et al. 2003
Wheat	Xylanase Glucanase	Layers	~2% (FCR, dose dependent)	Lazaro et al., 2003
Rye	Xylanase Glucanase	Layers	~6% (FCR, dose dependent)	Lazaro et al., 2003
Barley	Xylanase Glucanase	Layers	~4% (FCR, dose dependent)	Lazaro et al., 2003
Wheat	Xylanase	Broilers	~6% (FCR); ~10% (BWG)	Scott, 2005
Wheat	Xylanase Glucanase	Broilers	~5% (FCR); ~5% (BWG)	Wang et al., 2005
Wheat	Xylanase	Broilers	~10% AMEn[4]	Shakouri and Kermanshahi, 2003
Triticale	Xylanase	Broilers	~3% AMEn	Shakouri and Kermanshahi, 2003
Mean response to enzyme addition			~6%	

BWG = body weight gain, FCR = feed conversion ratio

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[4] AMEn is apparent metabolisable energy corrected to nitrogen balance. It corrects AME for the energy associated with N excretion (urinary energy).

3.4 Effect of conditioning temperature on feed processing

A recent study by one of the authors demonstrated the effect temperature conditioning can have on wheat-based diets for broiler chickens (Cowieson et al. 2005). In this study, performance enhancements associated with xylanase addition were more pronounced in diets that had been conditioned at 90ºC compared with those that had been conditioned at 80ºC. Chemical characterisation of the diets revealed that thermal processing had increased viscosity of the diets. Furthermore, the addition of exogenous xylanase reduced both viscosity and the degree of polymerization of the arabinoxylan-based carbohydrates. Similar responses have been noted previously, where an optimal conditioning temperature of 85ºC was noted for wheat-based broiler diets and adverse effects of higher temperatures were apparent (Silversides and Bedford 1999). Furthermore, recently presented data by Scott (2005) showed similar effects on Canadian wheats, where pelleting increased digesta viscosity by approximately 17%, but was significantly reduced with enzyme addition.

These studies suggest that, in order to achieve a more consistent response to exogenous xylanase, conditioning temperature should be considered as an important factor. In addition, the survival rate of XIs in wheat during conditioning should also be investigated. Even though XIs are relatively stable, it is likely that some denaturation will occur when exposed to temperatures used in feed processing.

It can be concluded that although there is some variability in the response to exogenous xylanase, there is a great deal of evidence in the literature that shows that these enzymes are effective in enhancing performance of poultry. However, although the inherent energy value of the wheat and the design of the diet explain most of the variance in response to exogenous xylanase, some of the variation may be explained by differences in the concentration of XIs in the grain.

3.5 Non-feed applications

It is important to note that even though the amount of feed wheat produced and consumed by poultry annually is considerable (6.3 million tonnes for the UK in 2004), the impact endogenous XIs present in wheat have on exogenously added enzymes is likely to be similar for the bread making industry. Even though in volume less bread making wheat is produced (approx. 3 million tonnes in 2004), a premium is paid for high quality wheat, and the enzyme market for food and beverages is also bigger than that for animal feed. During the bread making process, the flour (white and wholemeal) is subjected to higher moisture contents than when processed for poultry feed, and the duration of potential contact between XIs and added xylanases is also significantly

greater during bread making than during feed processing. Both higher moisture content and prolonged exposure means there is increased potential for contact between XI and xylanase.

3.6 Future developments in enzyme technology

Enzyme companies are always looking for new or improved products, and therefore xylanases with less or no interference from endogenous XIs would be potential candidates. Even though xylanases uninhibited by wheat XIs are known (*A. aculeatus* xylanase, wheat endogenous xylanases), they often do not meet other essential criteria needed to be used as feed supplement. Most importantly, because xylanase is only relatively briefly in contact with its substrate in the case of poultry feed, enzymes need to be highly active in order to be considered as feed improvers. This will be discussed in more detail in section 4.2. Other ways of reducing or eliminating potential XI impact on enzyme performance are also considered.

One interesting new area of research for xylanase in wheat-based diets is the role of enzyme molecular architecture in its affinity for soluble and insoluble fibre and the susceptibility to inhibition. For instance, a xylanase engineered with a carbohydrate-binding module was approximately 5% and 6% more efficacious in a wheat- and rye-based diet respectively compared with a commercial enzyme preparation that did not have the ability to attach to its substrate (Fontes et al. 2004). The authors conclude that the ability of enzymes to bind to their substrate increases the efficacy of the enzyme *in vivo*, especially associated with the hydrolysis of insoluble carbohydrate. Therefore, it is likely that this mode of action will allow a greater degree of cell wall hydrolysis, thereby releasing encapsulated nutrients. It may have even greater significance in diets high in NSPs (e.g. for grower/finisher pig rations).

4. Quantification of the impact on xylanase inhibitors on poultry feed performance and its value to the industry

To understand how the XIs present in feed wheat may affect FCE in poultry fed on wheat-based diets with exogenous xylanases, it is important to investigate how levels of xylanase inhibition relate to wheat AME. The impact of XIs may be greater in feed wheat with low AME, where the effect of xylanase addition is greater (see section 2.3). It is unlikely that XIs directly affect the nutritional quality of the wheat, since there are no substrates for the inhibitors present in the GI tract of broilers. Endogenous xylanases present in wheat are unaffected by endogenous XIs (Elliot et al. 2003).

Knowledge of the levels of endogenous xylanase inhibition present in UK wheat varieties may lead to the identification of wheat varieties low in XIs, and could result in more strategic use of exogenous enzymes. It also has potential to result in significant improvements in the cost-effectiveness of exogenous enzyme use, when used in combination with low-XI wheat.

4.1. Estimated impact of xylanase inhibitors on poultry feed nutrient content

The feeding of whole grain to poultry, whether in pelleted form or as scattered seeds, has certain implications for the commercial feed and poultry industry. As described previously, water-soluble and insoluble AX in the poultry diet resulting from whole grain inclusion greatly increases the anti-nutritive effects in the feed, which are counteracted by the now routine addition of exogenous xylanases to the feed.

4.1.1. Implications for animal health

As described previously, depending on the kind of wheat and xylanases used, broiler chicks fed on xylanase-supplemented diets gain significantly more weight, the digesta viscosity in their gut is reduced, as is the moisture level of the excreta (Choct et al. 2004). Therefore, addition of xylanase also has a significant impact on the welfare of the flock and the presence of XIs potentially reduces the benefits associated with xylanase addition.

4.1.2. Implications for Feed Conversion Efficiency (FCE)

Increasing xylanase activity by removing inherent xylanase inhibition in the feed should theoretically improve FCE and may result in a higher turnover of flocks per year. A calculated example based on FCE is given below. All values used in this example represent averages for broilers grown in the UK; variation will exist between producers and between flocks.

For poultry, an improvement of 1% in FCE will generally decrease feed intake per bird by 32g to achieve the same final bodyweight as a broiler currently grown to 42 days of age (in this example, 49 days per broiler are counted to include cleaning between flocks). Or, the broiler would need less time to reach its final bodyweight if FCE improves by 1%. Addition of xylanases improves FCE by 2.5%, and this is assuming that XIs are present in the feed.   The impact of xylanase inhibition can be calculated as follows:

By removal of xylanase inhibition (assuming a variation between 10 – 60% and addition of xylanases), FCE could theoretically be improved up to 2.75 – 4.05%. This means that broilers would reach their final weight quicker, and potentially more flocks could be grown per year. Typically, an average of 4500g of feed is consumed per broiler to reach its end weight, containing 2700g wheat. A 2.75% improvement in FCE would mean a broiler would consume 4412g of feed (2647g wheat) to reach its final weight in 48 days (inclusive of 7 days cleaning between flocks). A 4.05 % improvement in FCE would mean a broiler would consume 4370g of feed (2622g wheat) to reach its final weight in 47.6 days (inclusive of 7 days cleaning between flocks). In one year (365 days), potentially 7.45 flocks can be grown. According to Defra statistics (Defra 2005), last year 860 million broiler chicks were placed in the UK, thus around 11.5 million broilers per 'flock' on a national level. Therefore, improved FCE due to lower XI activity in feed wheat could increase broiler placings to 877 million annually (FCE up to 2.75%) or to 886 million annually (FCE up to 4.05%), an increase of 1.96% – 2.98%. A similar increase in feed wheat demand would have an annual value of £11 - £15 million (based on 2005 wheat prices).

In addition, the improved health and welfare of the broilers would also be a significant factor, as would a potential reduction in cleaning efforts between flocks due to dryer, more compact litter.

4.2. Approaches to improving poultry feed digestibility by reducing or eliminating XI interference

Assuming XIs do interact with added xylanases, and FCE and animal welfare could be improved by inactivating or removing them, there are three possible ways of doing so:

(1) by modifying the xylanases used in feed (so that they are not inhibited by endogenous XIs),

(2) by modifying feed processing methods (XI inactivation), or

(3) by modifying the wheat source used in feed (XI reduction).

4.2.1. New enzyme development

New enzyme development is a slow and expensive process, and enzymes have to go through lengthy trials to determine toxicity, efficacy etc. Even though enzyme companies are developing xylanases that are not inhibited by XIs, such an enzyme needs to meet several other criteria for efficient use in all applications. Examples of such criteria, apart from no inhibition by all XIs present in wheat, would include high specific activity and the relative cost of registering such an enzyme as a feed supplement. In some cases, a theoretically inhibited enzyme with proven high specific activity could be preferred to a relatively slow acting, uninhibited enzyme as the time that enzyme and substrate are into contact with each other (in the GI tract) is a limiting factor. Therefore, knowing the extent to which XIs are interfering with xylanase efficiency and how it affects AME would be paramount before further developing new enzymes.

4.2.2. Improvements in processing technology

Xylanase inhibitors present in wheat are probably not fully denatured during the pelleting procedures currently used in the feed industry (see below), although this has not been conclusively shown. By subjecting wheat to higher temperatures, e.g. 100°C for more than 15min, the activity of endogenous xylanase inhibitors may be reduced to undetectable levels, and performance of xylanases added after this process would be uninhibited. By changing the way poultry feed is processed, this extra heating step may reduce the effect of xylanase inhibitors significantly. However, there are several major drawbacks to this method. Firstly, as discussed in section 3.3, increasing temperature can increase feed viscosity. Additional xylanase may counteract it, but this would represent two additional costs. Secondly, currently feed is subjected to heat during grinding of the wheat and pelleting, and during the latter temperatures of 80-90°C for 1-1.5 minutes are reached. Increasing temperature up to 100°C and increasing the time of heat treatment will denature more than the XIs. Other proteins, vitamins, enzymes etc. also denature when exposed to such temperatures. In wheat-based feed (typically containing 65% wheat), the 11% crude protein derived from the wheat component would contribute 35.75% of the protein requirement, of which 17.05% of the lysine and 21.25% of the methionine (R. Clark-Monks, BPC, *pers. comm.*). Therefore, denaturing wheat protein would increase the protein requirement of the feed significantly. Exogenous xylanases could only be added after the pelleting process, as adding them before would similarly denature them. Finally, the most important drawback would most likely be the significant cost increase resulting from increasing feed processing temperature. Therefore, overcoming xylanase inhibition by increasing heat exposure during feed processing seems an unlikely option.

Another option is to use the endogenous xylanases present in the wheat to degrade the AX in the feed. Endogenous xylanases are not inhibited by endogenous XIs (Elliot et al. 2003). Scott (2003) showed that feeding dry pre-germinated grain to broilers results in a marked increase in feed intake and growth. Endogenous xylanases, which increase 10-fold during germination (Elliot et al. 2003), had removed the need for additional xylanases in the feed. This seems a simple solution in theory. However, designing and evaluating two extra steps to current feed processing procedures (pre-germinating and subsequent drying of wheat) would again increase costs significantly, and the nutritional value of feed thus processed would have to be re-established.

As illustrated by these two examples, it is unlikely that poultry producers would benefit from changing current feed processing methods to eliminate XIs.

4.2.3. Improvements in feed wheat varieties used

A third approach, described below, addresses the problem of XI activity in wheat from a wheat-genotype point of view. It has been shown from research in Belgium and France that the types, amounts and specificities of XIs present in wheat can differ significantly depending on the variety of wheat tested (Table 1, Figure 3). Similar research on UK-grown wheat could result in isolation of one or more wheat varieties currently grown for poultry feed with inherent low amounts of XIs. Feed trials would be needed to test whether these selected varieties have improved wheat AME and improve overall FCE in broilers. No extra processing step(s) would be needed during the poultry feed manufacturing process. If feeding trials show the benefits of reduced XI levels in poultry production, breeding programmes to select for wheat with very low or no XI activity could be established to produce such varieties of feed wheat. Again, the cost effectiveness of such a breeding programme *vs.* cumulative benefits would first have to be determined.

4.3. Assessment of laboratory methods needed to screen for xylanase inhibitor activity

Earlier sections (2.5-2.6) discussed the levels of xylanase activity reported in continental European wheats. The only way to gain similar information of relevance to UK wheats, is to carry out a screening programme.

4.3.1 Simple screens

Initially, identification of XIs present and their affinity towards different xylanases may not be needed. A quick and simplified screen for total XI activity in different feed wheats would provide a rough estimate of the potential problem. However, if crude screening of total XI activity in several UK feed wheats shows significant variation, then a more detailed investigation can be done. Several different methods to assess the level of xylanase inhibition have been published. Most methods rely

on comparing xylanase activity with and without inhibitors present, and express xylanase inhibition accordingly. Table 3 summarises the details of three previously published xylanase inhibition assays.

4.3.2 Screens for levels of specific XI activities

It is possible to use the differences in xylanase inhibition to quantify the different inhibitors. For example, if *A. nidulans* xylanase is used, inhibition by XIP-I can be estimated as this enzyme is not inhibited by TAXI. Similarly, *B. subtilis* xylanase can be used in an assay for quantification of TAXI, as this xylanase is not inhibited by XIP. However, to know to what extent these two xylanases are inhibited by their relevant inhibitors, purified enzymes and inhibitors are needed. It should also be noted that certain moulds such as *Aspergillus* (which is also used to produce commercial enzymes) sometimes present on the grain, also produce xylanases. This should be taken into account if detailed XI screening needs to be considered. Reducing contamination of such moulds is necessary to prevent underestimation of the xylanase activity when added to wheat, and consequently the quantification of the inhibitor.

Table 3. Comparison of assay methods for xylanase inhibition in cereals.

Reference	Principle	Minimal volume	Detection	Unit of measurement	Estimated time
Debyser et al. (1997)	Activity of xylanase solution with or without inhibitors (endosperm extract or known inhibitor solution) is measured. Xylazyme AX tablets (Megazyme, Bray, Ireland) are used as substrate for xylanase. Samples are incubated, then stopped, filtered and absorbance is measured at 590 nm.	Extract: 5 ml Assay: 1-2 ml (cuvette)	Spectro-photometer at 590 nm	1 Inhibition Unit (InU) is the amount of inhibitor necessary to reduce fixed xylanase activity by 50 %.	Extraction: 45 min. Assay: <= 2.5h
Bonnin et al. (2005)	Xylanases and inhibitors are purified from different sources. Inhibition using water-soluble AX is measured by comparing xylanase activity with and without inhibitor, using an alkaline $CuSo_4$ method for measuring reducing ends (Nelson 1944).	Assay: using a microplate: 20 μl, using a cuvette: 1-2 ml.	Spectro-photometer at 600 nm	1 nkat: the amount of enzyme necessary to release 1 nmole of reducing ends per second under the assay conditions. For quantification, dilutions do not exceed 50 % inhibition.	Purification of xylanases, inhibitors: several days. Crude extract: ½ day; assay: < 1h
Bailey et al. (1992)	Xylanase inhibitors are purified from different sources. Inhibition is measured by comparing xylanse activity with and without inhibitor, using the dinitrosalicylic acid (DNS) assay[5], using low viscosity arabinoxylan as substrate. After incubation and termination (DNS and boiling), absorbance of the supernatant is measured at 550 nm.	Assay: using a microplate: 20 μl, using a cuvette: 1-2 ml.	Spectro-photometer at 550 nm.	One unit of xylanase activity: the amount of protein that releases 1 μmol xylose per min at 30 °C and pH 5.5. The $E:I_{50}$ value is the molar ratio of enzyme – inhibitor required to inhibit xylanse activity by 50 %.	Purification of xylanase inhibitors: several days. Assay: enzyme dependent, >= 40 min.

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[5] It should be noted that according to Jeffries (1994), the DNS method may result in high values when employed for xylan assays. The DNS assay may over-estimate xylanase activity by threefold or more. **Jeffries TW**: Biodegradation of lignin and hemicelluloses. In *Biochemistry of microbial degradation*. Edited by Ratledge C: Kluwer Academic Publishers; 1994:233-277.

5. Cost assessment of possible laboratory methods for wheat screening

Based on the assay optimised by Gebruers et al. (2002), cost assessment of a quick screening of XI activity in 40 UK-grown wheat varieties is as follows:

Forty wheat grain extracts, each in duplicate, means 80 extracts containing XIs. Xylanase activity is measured with and without (control) extract, using the Megazyme endo-1,4-β-xylanase assay. Each assay should also be performed in duplicate, therefore to screen 40 wheat cultivars for xylanase inhibition, 162 assays need to be performed. The control assays only need to be duplicated once as that is the same for each extract, unless the results vary considerably. The kit contains 2 types of enzymes, one fungal and one bacterial, both of which have been shown to be inhibited by XIP- and TAXI-type inhibitors to varying degrees. Therefore, screening can be done with one or both enzymes, depending on the detail of information required. In the latter scenario, 162 assays can be performed in approx. 5 days excluding assay optimisation, which is assumed to be minimal but may add another 2-3 days. The cost would need to include the cost of acquiring the Megazyme kits. These amounts can be doubled if both enzymes are used separately to test levels of xylanase inhibition (384 assays).

6. Conclusions and recommendations

XIs are most likely present and active in all wheat varieties grown in the UK, and they interact with exogenous xylanases commercially available and added to wheat in many industrial applications to reduce viscosity or hydrolyse xylan chains. However, the true extent of variation due to genotypic and environmental factors has not been studied from a UK perspective, nor has their impact on xylanase performance in numerous industrial applications been quantified.

Based on the findings outlined in this report, it is recommended that the following should be carried out:

1. Initial lab-based screening of UK feed wheat varieties to determine XI levels. This would be relatively quick and informative, and results of such a screen would dictate further, more detailed research (recommendations 2-5);

2. Quantification of the impact of xylanase inhibitors using physiochemical tests (e.g. determining effects on viscosity directly) rather than reliance only on spectrophotometric assays;

3. Study of the contribution of fungal residue contamination potentially present on grains, which might have resulted in underestimation of the impact of xylanase inhibition in the past;

4. Study of the effect of feed processing at different temperatures, water contents, pressures and total diet composition on the survival of the inhibitors, and the nutritional value of the feed in relation to actual XI activity;

5. Quantification of xylanase performance in the presence and absence of XIs using feeding trials with young birds.

7. Literature cited

Adeola O, Bedford MR (2005). Exogenous dietary xylanase ameliorates viscosity-induced anti-nutritional effects in wheat-based diets for White Peking Ducks (*Anas platyrinchos). British Journal of Nutrition*, **92**:87-94.

Arro M, Richard L, Tran Thanh Van K, Ferrer A, Boronat A (1997). Cloning and characterization of a novel cDNA encoding thaumatin-like protein PR5 from *Arabidopsis thaliana* (Accession No. L78079). *Plant Physiology*, **113**:1463-1465.

Beavis W (1995). A standard for maize genetic nomenclature. *Maize Genetic Cooperation Newsletter*, **69**:182-184.

Bedford MR (2000). Exogenous enzymes in monogastric nutrition - their current value and future benefits. *Animal Feed Science and Technology*, **86**:1-13.

Bedford MR: The role of carbohydrases in feedstuff digestion. In *Poultry Feedstuffs: Supply, Composition and Nutritive Value*. Edited by McNab J, Boorman N: CAB International; 2002:319-336.

Bedford MR, Classen HL (1992). Reduction of intenstinal viscosity through manipulation of dietary rye and pentosanase concentration is effected through changes in the carbohydrate composition of the intestinal aqueous phase and results in improved growth rate and food conversion efficiency of broiler chicks. *Journal of Nutrition*, **122**:560-569.

Bonnin E, Daviet S, Gebruers K, Delcour JA, Goldson A, Juge N, Saulnier L (2005). Variation in the levels of the different xylanase inhibitors in grain and flour of 20 French wheat cultivars. *Journal of Cereal Science*, **41**:375-379.

Brutus A, Villard C, Durand A, Tahir TA, Furniss C, Puigserver A, Juge N, Giardina T (2004). The inhibition specificity of recombinant *Penicillium funiculosum* xylanase B towards wheat proteaceous inhibitors. *Biochimica et Biophysica Acta*, **1701**:121-128.

Choct M, Cocher A, Waters DL, Pettersson D, Ross G (2004). A comparison of three xylanases on the nutritive value of two wheats for broiler chickens. *British Journal of Nutrition*, **92**:53 - 61.

Collins T, Gerday C, Feller G (2005). Xylanases, xylanase families and extremophoilic xylanases. *FEMS Microbiology Reviews*, **29**:3-23.

Coutinho PM, Henrissat B: (1999) Carbohydrate-Active Enzymes (CAZy) server at URL: http://afmb.cnrs0mrs.fr/CAZY/.

Cowieson AJ, Hruby M, Faurschou Isaksen M (2005). The effect of conditioning temperature and exogenous xylanase addition on the viscosity of wheat-based diets and the performance of broiler chicks. *British Poultry Science*, ***in press***.

Debyser W, Delcour JA, Derdelinckx G (1997). Arabinoxylan Solubilization and Inhibition of the Barley Malt Xylanolytic System by Wheat During Mashing with Wheat Wholemeal Adjunct: Evidence for a New Class of Enzyme Inhibitors in Wheat. *Journal of the American Society of Brewing Chemists*, **55**:153-156.

Debyser W, Peumans WJ, Van Damme J, Delcour JA (1999). *Triticum aestivum* xylanase inhibitor (TAXI), a new class of enzyme inhibitor affecting breadmaking performance. *Journal of Cereal Science*, **30**:39-43.

Defra: Poultry and Poultrymeat Statistics Notice. Edited by: Defra; 2005.

Durand A, Hughes R, Roussel A, Flatman R, Henrissat B, Juge N (2005). Emergence of a subfamily of xylanase inhibitors within glycoside hydrolase family 18. *FEBS Journal*, **272**:1745-1755.

Elliot GO, McLauchlan WR, Williamson G, Kroon PA (2003). A wheat xylanase inhibitor protein (XIP-I)accumulates in the grain and has homologues in other cereals. *Journal of Cereal Science*, **37**:187-194.

Fontes CM, Ponte PI, Reis TC, Soares MC, Gama LT, Dias FM, Ferreira LM (2004). A family 6 carbohydrate-binding module potentiates the efficiency of a recombinant xylanase used to supplement cereal-based diets for poultry. *British Poultry Science*, **45**:648-656.

Frederix SA, Courtin CA, Delcour JA (2004). Substrate specificity and inhibitor sensitivity affect xylanase functionality in wheat gluten-starch separation. *Journal of Cereal Science*, **40**:41-49.

Gebruers K, Courtin CM, Goesaert H, Van Campenhout S, Delcour JA (2002). Endoxylanase inhibition activity in different European wheat cultivars and milling fractions. *Journal of Cereal Chemistry*, **79**:613-616.

Gebruers K, Debyser W, Goesaert H, Proost P, Van Damme J, Delcour JA (2001). *Triticum aestivum* L. endoxylanase inhibitor (TAXI) consists of two inhibitors, TAXI I and TAXI II, with different specificities. *Biochemical Journal*, **353**:239-244.

Gebruers K, Fierens K, Rombouts S, Courtin CM, Brijs K, Volckaert G, Van Campenhout S, Delcour JA: TL-XI, a novel type of xylanase inhibitor from wheat belonging to the thaumatin-like protein family. In *AACC/TIA Joint Annual Meeting*; *San Diego, California, US*: 2004.

Gebruers K, Brijs K, Courtin CM, Fierens K, Goesaert H, Rabijns A, Raedschelders G, Robben J, Sansen S, Sørensen JF, et al. (2004). Properties of TAXI-type endoxylanase inhibitors. *Biochimica et Biophysica Acta*, **1696**:213-221.

Goesaert H: Occurrence, purification and properties of endoxylanase inhibitors in different cereals: Ph.D. thesis, Katholieke Universiteit Leuven, Belgium: 2002.

Goesaert H, Gebruers K, Brijs K, Courtin CA, Delcour JA (2003a). TAXI type endoxylanase inhibitors in different cereals. *Journal of Agricultural and Food Chemistry*, **51**:3770 - 3775.

Goesaert H, Gebruers K, Brijs K, Courtin CA, Delcour JA (2003b). XIP-type endoxylanase inhibitors in different cereals. *Journal of Cereal Science*, **38**:317 - 324.

Graham H, Simmins PH, Sands JS (2003). Reducing environmental pollution using animal feed enzymes. *Communications in Agriculture and Applied Biological Science*, **68**:285-289.

Hayes TL: Industry Study 1949: World Enzymes. Edited by. The Freedonia Group Inc., Cleveland, Ohio; 2005:132-136.

Igawa T, Ochiai-Fukuda T, Takahashi-Ando N, Ohsato S, Shibata T, Yamaguchi I, Kimura M (2004). New TAXI-type Xylanase Inhibitor Genes are Inducible by Pathogens and Wounding in Hexaploid Wheat. *Plant and Cell Physiology*, **45**:1347-1360.

Ingelbrecht JA, Verwimp T, Delcour JA (2000). Endoxylanases in durum wheat semolina processing: solubilization of arabinoxylans, action of endogenous inhibitors, and effects on rheological properties. *Journal of Agricultural and Food Chemistry*, **48**:2017-2022.

Jeffries TW: Biodegradation of lignin and hemicelluloses. In *Biochemistry of microbial degradation*. Edited by Ratledge C: Kluwer Academic Publishers; 1994:233-277.

Jeffries TW: (1996) Biochemistry and genetics of microbial xylanases; http://www2.biotech.wisc.edu/jeffries/xylanase_review/xyl_rev.html.

Juge N, Payan F, Williamson G (2004). XIP-I, a xylanase inhibitor protein from wheat: a novel protein function. *Biochimica et Biophysica Acta*, **1696**:203-211.

Lazaro R, Garcia M, Aranibar MJ, Mateos GG (2003). Effect of enzyme additino to wheat-, barley- and rye-based diets on nutrient digestibility and performance of laying hens. *British Poultry Science*, **44**:256-265.

Mathlouthi N, Juin H, Larbier M (2003). Effect of xylanase and beta-glucanase supplementation of wheat-or wheat- and barley-based diets on the performance of male turkeys. *British Poultry Science*, **44**:291-298.

Mathlouthi N, Mohamed MA, Larbier M (2003). Effect of enzyme preparations containing xylanase and beta-glucanase on performance of laying hens fed wheat/barley- or maize/soybean meal-based diets. *British Poultry Science*, **44**:60-66.

McLauchlan WR, Garcia-Conesa MT, Williamson G, Roza M, Ravenstein P, Maat J (1999). A novel class of protein from wheat which inhibits xylanases. *Biochemical Journal*, **338**:441-446.

Meng X, Slominski BA, Nyachoti CM, Campbell LD, Guenter W (2005). Degradation of cell wall polysaccharides by a combination of carbohydrase enzymes and their effect on nutrient utilization and broiler chicken performance. *Poultry Science*, **84**:37-47.

Nelson N (1944). A photometric adaptation of the Somogyi method for the determination of glucose. *Journal of Biological Chemistry*, **153**:375 - 380.

Nilsson M, Andersson R, Andersson RE, Autio K, Aman P (2000). Heterogeneity in water-extractable rye arabinoxylan with a low degree of distribution. *Carbohydrate Polymers*, **41**:397-405.

Ponte PIP, Ferreira LMA, Soares MAC, Gama LT, Fontes CMGA (2004). Xyanase inhibitors affect the action of exogenous enzymes used to supplement *Triticum durum*-based diets for broiler chicks. *Journal of Applied Poultry Research*, **13**:660-666.

Rouau X, Surget A (2002). Evidence for the presence of a pentosanase inhibitor in wheat flours. *Journal of Cereal Science*, **28**:63-70.

Plant Breeding Updates on World Wide Web URL: www.desicca.de/plant_breeding

Scott TA: A possible explanation for limited feed intake of wheat based diets by broilers. Edited by. Sydney: Poultry Research Foundation, University of Sydney; 2003.

Shakouri MD, Kermanshahi H (2005). Effect of NSP degrading enzyme supplement on the nutrient digestibility of young chickens fed wheat with different viscosities and triticale. *Journal of Agricultural and Scientific Technology*, **5**:105-112.

Sieo CC, Abdullah N, Tan WS, Ho YW (2005). Influence of glucanase-producing *Lactobacillus* strains on intestinal characteristics and feed passage rate of broiler chickens. *Poultry Science*, **84**:734-741.

Silversides FG, Bedford MR (1999). Effect of pelleting temperature on the recovery and efficacy of a xylanase enzyme in wheat-based diets. *Poultry Science*, **78**:1184-1190.

Sørensen JF, Kragh KM, Sibbesen O, Delcour JA, Goesaert H, Svensson B, Tahir TA, Brufau J, Perez-Vendrell AM, Bellincampi D, et al. (2004). Potential role of glycosidase inhibitors in industrial biotechnological applications. *Biochimica et Biophysica Acta*, **1696**:275-287.

Trudel J, Grenier J, Potvin C, Asselin A (1998). Several thaumatin-like proteins bind to the beta-1,3-glucans. *Plant Physiology*, **118**:1431-1438.

Van der Kils JD, Van Voorst A, Van Cruyningen C (1993). Effect of a soluble polysaccharide (carboxy methyl cellulose) on the physico-chemical conditions in the gastrointestinal tract of broilers. *British Poultry Science*, **34**:971-983.

Watanabe T, Kobori K, Miyashita K, Fujii T, Sakai H, Uchida M, Tanaka H (1993). Identification of glutamic acid 204 and aspartic acid 200 in chitinase A1 of Bacillus circulans WL-12 as essential residues for chitinase activity. *J. Biol. Chem.*, **268**:18567-18572.

Weightman RM, Forge CD, Quandalle C (2001). A rapid viscometric screening tool for measuring feed wheat quality and the relationship between the quality of hybrid wheats and their parental lines. *Aspects of Applied Biology*, **64**:79-84.