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SYMPOSIUM ON 'APPLICATION OF BIOTECHNOLOGY TO LIVESTOCK PRODUCTION'

Biotechnology and improvement of feeds

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A high proportion of barley and wheat grown in Western Europe and maize in the USA is used as feed for livestock. When used for simple-stomached animals, such as pigs and poultry, the nutritional quality is limited by the low amounts of certain essential amino acids, and it is necessary to supplement the diet with sources of these amino acids for maximum growth. The definitive studies of Fuller *et al.* (1979*a*,*b*) have shown that barley is deficient in lysine, threonine and (marginally) histidine as feed for pigs, and it can be assumed that the same applies to wheat. For maize the limiting amino acids are lysine and tryptophan (Bressani, 1975).

The present discussion will be restricted to barley, and will concentrate on studies from our own laboratory. We will also consider feed quality as the proportions of lysine and threonine in the grain proteins, although we appreciate that this definition ignores the important factor of protein digestibility. Furthermore, since lysine is the first limiting amino acid, and proteins which contain a high proportion are generally also rich in threonine (and vice versa), the emphasis will be on lysine content.

The biochemical description of barley seed quality can be applied, with relatively minor alterations, to wheat and maize. However, because wheat is a hexaploid, the application of this information to improvement will be more difficult than with the diploids barley and maize.

Barley seed proteins: the major determinants of feed quality

The proteins of plant seeds are usually classified into groups based on their extraction and solubility in a series of solvents: water (albumins), dilute aqueous solutions of salts (globulins), aqueous alcohols (prolamins) and dilute acids or alkalis (glutelins). This classification is based on work carried out around the turn of the century by T. B. Osborne (see Osborne, 1924), and has formed the basis for subsequent studies. In barley (and most other cereals including wheat and maize) the prolamins are the major storage proteins, while the other fractions each consist of a mixture of structural and metabolic proteins, possibly with some minor storage proteins. Thus it is convenient for the purpose of discussion to classify the seed proteins into only two groups: prolamins and others. This classification is also valid in relation to quality. Whereas the prolamins contain less than 10 mmol lysine/mol and about 25 mmol threonine/mol, the other fractions contain about 40–50 mmol of each per mol.

In addition to these groups of proteins, the grain also contains small amounts of free amino acids. In the case of lysine and threonine these represent 1% or less of the total amounts present in the seed.

We will now consider the properties of these fractions in more detail, and discuss strategies for manipulating their amounts or compositions. We will finally discuss the current technical limitations to the applications of these approaches.

The structure and manipulation of barley prolamins (hordeins)

Hordein is a mixture of polypeptides which can be divided into three groups called B, C and D hordeins. These together account for about 35–50% of the total nitrogen of the mature grain. The B and C hordein groups account for about 80–90% and 10–20% respectively of the total fraction and exhibit a high degree of polymorphism within and between cultivars. Genetic analysis shows that these groups are encoded by linked structural loci, designated *Hor1* (C hordein) and *Hor2* (B hordein) on the short arm of chromosome 5 (see Shewry & Miflin, 1982). Molecular analyses (Shewry *et al.* 1985; Bunce *et al.* 1986) show the presence of about twenty to thirty copies per haploid genome at each locus, which is sufficient to suggest that each polypeptide is encoded by one or more genes.

The polypeptides encoded by each locus have a high degree of structural homology (see Faulks *et al.* 1981; Shewry *et al.* 1981; Kreis *et al.* 1987). The number of lysine residues present in C hordein is probably only from zero to two per molecule of about 440 residues (Shewry *et al.* 1981). Similarly B hordeins contain one or two lysine residues per molecule of about 250–275 residues (see Kreis *et al.* 1987).

D hordein is only a minor component, and consists of either one or a small number of polypeptides encoded by a small multigene family (one or two copies) at the *Hor3* locus on chromosome 5 (Shewry *et al.* 1983; Bunce *et al.* 1986). It contains about 1% lysine (Field *et al.* 1982). Because D hordein has little effect on overall grain quality, it will not be considered further here.

Because the low contents of lysine in B and C hordeins are largely responsible for the poor nutritional quality of the whole grain, these groups have been the major focus of attempts to improve quality. Two types of strategy have been suggested.

1. Decreasing the proportion of hordein in the grain. This is the major effect in a number of induced high-lysine mutants of barley, including the Risø series (Doll, 1983). This decrease is accompanied by increases in other more lysine-rich proteins, and in some cases also in free amino acids. The reduction of hordein varies from about 65% (in Risø 1508) to less than 10% (in Risø 7). In all cases except one the high-lysine phenotype results from a mutation at a 'regulatory' locus, which is not closely linked to the hordein structural loci. The exception is Risø 56, in which the mutation has been shown to involve the deletion of most if not all of the genes at the *Hor2* locus (Kreis *et al.* 1983). The disadvantage of all these high-lysine mutations (including Risø 56) is that they also have pleiotropic effects on other aspects of seed metabolism, including the synthesis of starch (and hence yield). The reasons for these effects are not known, and it has proved impossible to separate them from the high-lysine phenotype by conventional crossing or plant breeding programmes. It has been suggested that a high level of storage protein synthesis is essential for normal grain filling (Doll & Kreis, 1979). If so, it may be necessary to combine specific modification of hordein gene expression (perhaps by engineering of cloned regulatory genes) with increases in the levels of expression of other storage protein genes.

We have isolated a B hordein gene (Forde *et al.* 1985) and are currently studying its control of expression in vivo in barley, and in other plants such as tomato and tobacco

(Kreis *et al.* 1987). The information from studies of this type will be important if we eventually wish to manipulate the expression of hordein genes in developing endosperms.

The reader is referred to reviews by Doll (1983), Miflin & Shewry (1979) and Shewry *et al.* (1987) for further discussion of the mutant high-lysine genes.

2. Changing the amino acid composition of hordeins. Alteration of the hordein structural genes to encode proteins containing more residues of lysine and threonine is, on superficial consideration, a very direct and attractive approach. There are, however, two major problems: the extensive modifications that will be required, and the presence of multigene families encoding B and C hordeins. It can be calculated that at least ten lysine residues must be added to each hordein polypeptide to increase the content to a nutritionally acceptable level of 50 mmol/mol. In addition it will also be necessary to add threonine residues. Although it is possible to isolate the genes for hordeins (Brandt *et al.* 1985; Forde *et al.* 1985) and to increase the number of codons for lysine by mutagenesis or the insertion of additional sequences, this must be done without affecting the mode of deposition or digestibility of the protein. Although the latter may not be a problem, it may be necessary to insert the additional lysines in specific regions or as a tail at the N- or C-terminus.

The presence of multigene families poses a more serious challenge. It may be necessary to insert many genes modified as discussed above, or sufficient to insert only one or a small number under the control of strong promoter sequences. This can only be determined experimentally. In either case it will be necessary to use a recipient line in which the wild-type genes are deleted (such as Risø 56) for the insertion of manipulated B hordein genes. If Risø 56 is used, it will first be necessary to determine the origin of its reduced starch content and yield.

Increasing the amounts of lysine-rich proteins

Hejgaard & Boisen (1980) identified four high-lysine, salt-soluble proteins that contributed significantly to the increased lysine content of lines derived from Hiproly, a spontaneous high-lysine mutant from Ethiopia (Table 1). These are two chymotryptic

Table 1. *Amounts of β-amylase (EC 3.2.1.2), protein Z and chymotryptic inhibitors CI-1 and CI-2 present in normal and high-lysine barley genotypes, and lysine contents**

	High-lysine proteins (mg protein/g grain)				Total protein
Genotype	β-amylase	Protein Z	CI-1	CI-2	(mg/g grain)
Mona	0·98	2·2	0·24	0·08	100
Hiproly × Mona[5]	4·3	4·3	1·8	0.67	127
Bomi	0·85	2·5	0·23	0·06	98
Hiproly × Bomi[2]	3·2	4·5	1·7	0·38	113
Risø 1508	ND	0·5	≃1·5		ND
Risø 56	ND	4·0	ND		ND
Pirrka (normal genotype)	ND	0·2	ND		ND
Lysine content: g/kg	50	71	95	115	–

ND, not determined.

*Based on Hejgaard & Boisen (1980), Boisen *et al.* (1981) and Hejgaard (1982); see Shewry & Miflin (1985).

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Table 2. *Amounts of proteins Q, N, C and K present in eight barley genotypes, and lysine contents**

Proteins . . .	Q	N	C	K
Amounts present in eight genotypes (mg/g grain)	0·23–0·45	0·19–0·40	0·09–0·60	0·18–0·42
Lysine content (mmol/mol)	44	35	34	79

*From Hejgaard & Bjorn (1985).

inhibitors (CI-1 and CI-2), β-amylase (*EC* 3.2.1.2) and a protein of unknown function called protein Z. They together account for about 17% of the total lysine in the high-lysine lines compared with 7% in normal cultivars, and about half of the increased lysine content. There is also variation in the amounts of these proteins in other high-lysine mutants (Risø 1508, Risø 56) and in normal genotypes (Table 1) (see Shewry & Miflin, 1985; Shewry *et al.* 1987). Recently Hejgaard & Bjorn (1985) have characterized four further basic salt-soluble proteins, which they called C, K, N and Q. The amounts of these vary considerably between genotypes (Table 2), and they together account for up to 5% of the total salt-soluble proteins. Protein K is particularly rich in lysine (79 mmol/mol), and may represent about 1% of the total lysine present in the grain.

These eight proteins also act as storage proteins in that their amounts increase as extra nitrogen is supplied (Giese & Hejgaard, 1984). This, together with the observation that considerable fluctuations in their amounts can occur without any apparent effects on endosperm development, makes them attractive candidates for genetic engineering.

In order to understand the regulation of expression of the genes for these proteins we have isolated cDNA clones for CI-1 (M. S. Williamson and M. Kreis, unpublished results), CI-2 (Williamson *et al.* 1987) and β-amylase (M. Kreis, M. S. Williamson, B. Buxton, J. Pywell, J. Hejgaard and I. Svendsen, unpublished results). These are now being used as probes to isolate corresponding genes. Studies of the regulation of these genes in vivo and in vitro together with investigations of the mode of synthesis and deposition of the proteins (in collaboration with Dr N. Harris, University of Durham) will enable us to evaluate the potential for increasing the contents of the proteins in the grain by genetic engineering.

Table 3. *The contents of lysine, threonine and methionine in mature grains of four barley mutants and the wild-type parent (Bomi)**

	Amino acid content (nmol/g dry wt)		
Genotype	Threonine	Lysine	Methionine
Bomi	118	84	24
R2501	6129	1266	149
R2506	9032	1620	72
Bomi	127	79	19
R3004	2376	107	23
R3202	125	109	19

*Values taken from Bright *et al.* (1984).

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Increasing the amounts of free lysine and threonine

Bright *et al.* (1984, 1985) have described the selection of four induced mutants of barley that accumulate free amino acids in the mature grain. Two (R2501, R2506) have substantial increases in lysine and threonine (Table 3), which are sufficient to make small but significant contributions to the contents of these amino acids in the whole grain. These mutants also have small increases in methionine. The other two mutants, R3004 and R3202, have only small increases in lysine and no changes in methionine. They differ in their contents of threonine, which is increased substantially in R3004 but not in R3202.

Although these mutants may have reduced yield, Bright *et al.* (1985) ascribe this to linked secondary mutations, rather than pleiotropic effects of the same genes.

The increases in free amino acids in these mutants result from changes in the regulation of isoenzymes of aspartate kinase (*EC* 2.7.2.4), the first enzyme catalysing their synthesis from aspartate. Cloning and characterization of the genes for these enzymes in the wild-type and mutant forms will allow us to assess the potential for effecting further increases by genetic engineering. To achieve this it may also be necessary to isolate, characterize and modify the genes of other enzymes in the pathway, notably dihydrodipicolinic acid synthase (*EC* 4.2.1.52) (Bright *et al.* 1984, 1985).

Technical limitations to the genetic engineering of barley

The essential steps in the genetic engineering of a higher plant such as barley are summarized in Table 4. Plant genes can now be isolated routinely, provided there is enough information about their products to allow identification. This is the case for most barley-seed protein genes. Similarly, it is possible to make specific modifications to their regulatory or coding sequences using the techniques of protein engineering (for example, see Botstein & Shortle, 1985).

There are two major limitations of the application of these techniques to barley-grain quality. Firstly, it is not yet possible to re-insert the mutated genes into barley protoplasts or other cereal tissues, and to regenerate transformed plants. However, success has recently been achieved with the transformation of other cereals (Fromm *et al.* 1985, 1986; Lörz *et al.* 1985) and regeneration from rice protoplasts (Anderson, 1986) and similar procedures should be applied to barley in the near future. A more serious limitation is our lack of understanding of how the genes for barley-seed proteins are regulated, and the consequences of altering this regulation and the structures of the encoded proteins for the development of the endosperm. This can only be overcome by research of the type outlined previously.

Table 4. *Essential steps in the genetic engineering of plants*

Step	Application to barley-seed quality
1. Clone and characterize structural or regulatory gene	Several genes for seed proteins have been isolated; the next step will be the isolation of regulatory genes
2. Mutate or otherwise modify protein-coding or (*cis* or *trans* acting) regulatory sequences	Methods to carry out the modifications are currently available; more information is required on gene regulation and acceptable changes to protein structure
3. Introduce the new or altered sequence into the plant genome so that it is expressed in the desired manner	Cereal transformation and regeneration systems are being developed, but have not yet been applied to barley; more information is required on the regulation of expression of the introduced genes

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Prospects for barley-feed improvement

We believe that barley-seed quality can be improved by genetic engineering, but that more information is required to enable the most appropriate strategy (or combination of strategies) to be selected. Also, there are currently some technical limitations in the application of the procedures to barley. It appears to be important to maintain a high level of storage protein synthesis in the grain, so it may be necessary to combine decreases in total prolamins with increases in specific lysine-rich salt-soluble proteins. However, this will depend on the demonstration that the latter can be increased sufficiently without affecting grain development. Further increases in lysine may be achieved by combining these approaches with increases in the amounts of free lysine and threonine, by using the mutants identified by Bright *et al.* (1984) or by isolation and modification of the genes for aspartate kinase, other enzymes, or both.

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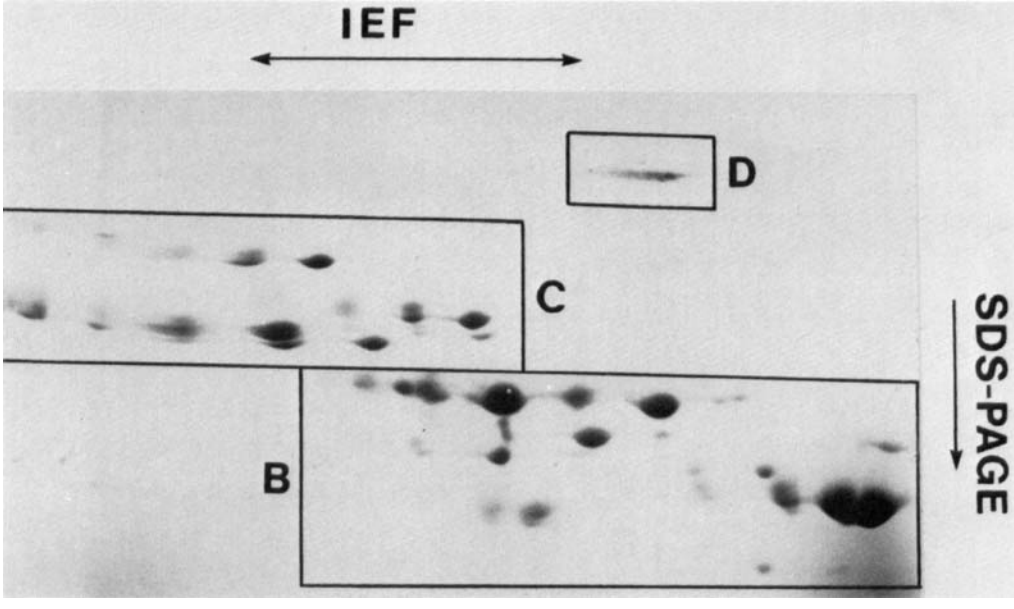

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EXPLANATION OF PLATE

Plate 1. Analysis of B, C and D hordeins by two-dimensional analysis and characteristics of the component groups of polypeptides (based on values from Shewry *et al.* 1980; Field *et al.* 1982; Kreis *et al.* 1984).

Hordein group	Molecular wt		Amino acid composition‡						
	SEU*	SDS–PAGE†	Glx	Pro	Gly	Phe	Cys	Met	Lys
D	54·7	105	300	120	160	14	15	2	11
C	52	59–72	410	310	3	88	0	2	2
B	32–35	36–45	350	210	15	48	25	6	5

Glx, glutamate+glutamine; Pro, proline; Gly, glycine; Phe, phenylalanine, Cys, cysteine; Met, methionine; Lys, lysine; IEF, isoelectric focusing.

*Molecular weight by sedimentation equilibrium ultracentrifugation.

†Molecular weight by sodium dodecylsulphate–polyacrylamide gel electrophoresis (SDS–PAGE).

‡Partial amino acid composition (mmol/mol).

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