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Multiple molecular forms of β-amylase in seeds and vegetative tissues of barley

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Abstract. The molecular forms of β-amylase present in developing, mature, germinating and malted grains of barley (*Hordeum vulgare* L.), and in vegetative tissues, have been studied using Western-blot analyses and isoelectric focusing of isoenzymes. Five isoforms with different relative molecular masses (M_rs) could be recognised. The major isoform present in the mature grain, called isoform B, had an M_r of about 60000. This was converted on malting or germination to two lower-M_r forms called C and D. Previous work (R. Lundgard and B. Svensson, 1986, Carlsberg Res. Commun. **51**, 487–491) has shown that these result from partial proteolysis of isoform B. Isoenzyme analyses showed complex patterns of bands, with pIs between about 5.0 and 6.0. Two allelic types were present in the eight lines. A number of new bands with a range of pIs appeared during germination and malting.

An isoform with the same M_r as D and a minor low-M_r isoform (E) were present in young developing whole caryopses (8–12 d after anthesis), but not in older developing endosperms (14–21 d after anthesis). Isoenzyme analyses also showed different patterns of bands in these two tissues, while hybrid-dot analyses indicated the presence of separate populations of mRNAs. It is suggested that the early endosperm isoforms (D and E) are "green" β-amylases present in the pericarp and-or testa of the young caryopses.

Roots but not shoots or leaves also contained an isoform with the same M_r as D, although the pattern of isoenzymes differed from that present in the seed tissues.

The fifth isoform, A, was a diffuse high-M_r form present in small amounts in all seed and vegetative tissues, and may correspond to a constitutively expressed form.

These multiple molecular forms of β-amylase are discussed in relation to the recent report that β-amylase is encoded by two structural loci, with a total copy number of two to three per haploid genome (Kreis et al., 1988, Genet. Res. Camb. **51**, 13–16).

Key words: β-Amylase (isoforms) – *Hordeum* (β-amylase) – mRNA populations – Seed (β-amylase)

Introduction

β-Amylase (1,4-α-D-glucan maltohydrolase) is a key enzyme in the mobilization of starch in germinating seeds of barley and other cereals. It is synthesised in the developing endosperm, where it is stored until germination. This contrasts with malt α-amylase which is synthesised de novo in the germinating aleurone and embryo (Briggs 1973). β-amylase resembles the major grain storage proteins (hordeins) in that its amount increases in response to added fertilizer nitrogen (Giese and Hejgaard 1984), and storage has been proposed as a secondary role. The amount of β-amylase in barley seeds is also affected positively or negatively by mutant "high lysine" genes, and in the former case may contribute to the increased proportion of lysine in the total seed proteins (see Shewry et al. 1987). Although only the seed form of the enzyme has been studied in detail, β-amylase is also present in vegetative tissues such as leaves (Levi and Preiss 1978).

A number of studies have shown that the β-amylase of mature and malted (i.e. germinated) grains exists in multiple forms. A free or soluble

Abbreviations: M_r = relative molecular mass; SDS-PAGE = sodium dodecyl sulfate-polyacrylamide gel electrophoresis

form is readily extracted in the absence of reducing agents, and can be separated by isoelectric focusing or chromatofocusing into at least eight isoenzymes with different isoelectric points (pIs) (LaBerge and Marchylo 1983; Ainsworth et al. 1987). This heterogeneity may result at least partly from the formation of aggregates with itself and with other proteins, including heterodimers with protein Z (Nummi et al. 1965; Hejgaard and Carlsen 1977; LaBerge and Marchylo 1983). The formation of aggregates stabilized by inter-chain disulphide bonds may also account for the origin of the bound form of β-amylase, which is only extracted in the presence of strong reducing agents. Once extracted and reduced the free and bound forms have identical relative molecular masses (M_rs) and immunological properties (Nummi et al. 1965; Hejgaard 1978). However, a further but still immunologically related form appears on germination or malting of the grain. This has a lower molecular weight and may derive from proteolytic modification of the grain form (Nummi et al. 1965; Hejgaard 1978).

In the present paper we use a recently characterized cDNA clone for β-amylase from barley seeds (Kreis et al. 1987) to study the populations of β-amylase mRNA in developing endosperms, and compare the molecular forms of the enzyme present with those in mature seeds, germinating seeds and vegetative tissues using isoenzyme analysis and Western blotting.

Materials and methods

Plant material. Barley (*Hordeum vulgare* L. cv. Sundance) was grown in the field at Rothamsted in 1987 and ears were labelled at anthesis. Ears were harvested at 8, 10, 12, 14, 16 and 21 d after anthesis. Endosperms were prepared from the 14- 16- and 21-d samples and whole caryopses from the earlier (8- 10- and 12-d) samples where it was not possible to prepare endosperms. This material was frozen in liquid N_2 and stored at −80° C. Leaves were harvested from glasshouse-grown seedlings of about eight weeks old, and roots and shoots from seedlings grown on sterile vermiculite at 20° C for one week.

To study changes in β-amylase during germination, 200 g of seed (cv. Sundance) were surface-sterilized and soaked in aerated water for 15 h at 20° C. They were then planted in sterile vermiculite and grown for 6 d at 20° C. Samples were removed after imbibition (called day 1) and then daily (days 2–7) and the seeds (after removal of roots and shoots) washed with distilled water and stored at −20° C. Undried commercial malt (cv. Maris Otter) was supplied by Dr. P. Blenkinsop (Hugh Baird and Sons (Malsters), Witham, Essex, UK) and stored at −20° C.

Mature dry seeds of other cultivars were from the collection at Rothamsted.

Extraction and analysis of proteins. Tissues were lyophilized and ground with a pestle and mortar. Proteins for Western-blot analysis were extracted by stirring for 2 × 1 h at 20° C with 10 ml/g of 0.1 M phosphate buffer, pH 8.0, containing 5 mM dithiothreitol (DTT) (Giese and Hejgaard 1984). The bulked supernatants were dialysed against distilled water, lyophilized and redissolved in an appropriate volume of 8 M urea containing 10% (w/v) sodium dodecyl sulphate and 10% (v/v) 2-mercaptoethanol. Free and bound forms were prepared by two extractions in the absence of DTT followed by two extractions in the presence of DTT.

Hordein fractions were extracted from developing endosperms, after extraction of salt-soluble proteins, with 50% (v/v) aqueous propan-1-ol containing 1% (v/v) acetic acid and 2% (v/v) 2-mercaptoethanol at 20° C (Shewry et al. 1983). The redissolved proteins were separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) using a modified Laemmli (1970) system with 14% (w/v) acrylamide and 4 M urea (Bunce et al. 1985). Western-blot analysis was carried out essentially as described by Kruger and Hammond (1988), but using 5% (w/v) freeze-dried low-fat milk as a blocking agent and protein-A alkaline phosphatase to detect the bound antibody. Antibodies against the grain form of β-amylase and the B1-hordein band (cv. Julia) were raised in rabbits (Festenstein et al. 1987; Hejgaard 1976) and used for Western-blot analysis at dilutions of 1:500.

The extraction of proteins and analysis of β-amylase isoenzymes by isoelectric focusing with starch as a substrate was carried out as described by Ainsworth et al. (1983). Because of the presence of α-amylase in some tissues, duplicate analyses were carried out after heating at 70° C for 10 min to inactivate β-amylase (Gale et al. 1983). β-amylase isoenzymes were also identified by using β-limit dextran instead of starch as a substrate.

Immunoprecipitation of β-amylase from the extracted proteins was carried out as described by Jonassen et al. (1981). The precipitated protein was redissolved and separated by SDS-PAGE as described above.

Extraction and analysis of mRNAs. The mRNA fractions were extracted from the developing grain and vegetative tissues and sequences related to β-amylase and B1 hordein detected by hybrid-dot analysis (as described by Kreis et al. 1987).

Results

β-Amylase in mature barley grains. Figure 1A (tracks a–h) shows SDS-PAGE analyses of proteins extracted from mature grain of eight barley lines with phosphate buffer in the presence of a reducing agent. These are five cultivars (Sundance, Pirrka, Bomi, Carlsberg II and Vada), one spontaneous high-lysine mutant (Hiproly) (Munck et al. 1970) and two induced high-lysine mutants (Risø 56 and Risø 1508, derived from Carlsberg II and Bomi respectively) (Ingversen et al. 1973; Doll et al. 1974; Karlsson 1976; Doll 1980). All except Risø 1508 (track e) have a major band of M_r about 60000 (arrowed in track a) which is precipitated by antiserum raised against seed β-amylase (Fig. 1, track i).

Western-blot analysis of the salt-soluble protein fraction from Sundance showed that the antiserum also reacted with a diffuse band of higher M_r (Fig. 2B, track g), and that both forms were pres-

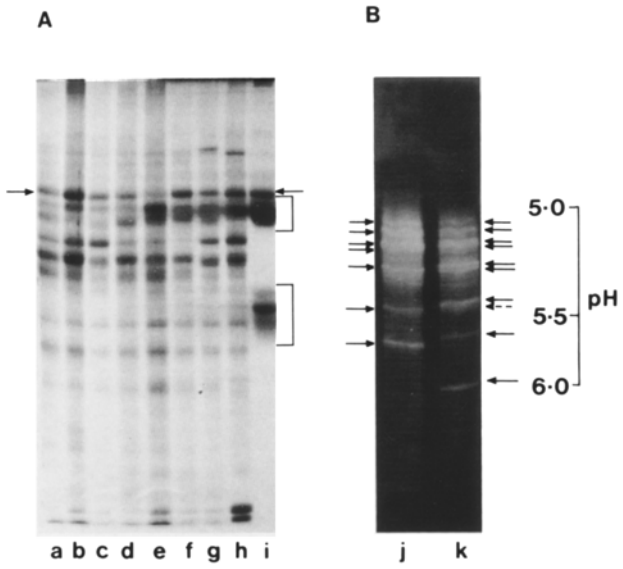

Fig. 1 A, B. β-Amylase in mature seeds of cultivars and mutant high-lysine lines of barley. **A** Analysis of total salt-soluble proteins by SDS-PAGE. *Tracks a–h* are from the following lines: *a*, Sundance; *b*, Risø mutant 56; *c*, Carlsberg II; *d*, Bomi; *e*, Risø mutant 1508; *f*, Pirrka; *g*, Vada; *h*, Hiproly. *Track i* is salt-soluble proteins of cv. Hiproly after immunoprecipitation with anti-serum to β-amylase. The *arrows* in *tracks a* and *i* indicate the major β-amylase band. The *brackets* in *track i* indicate the immunoglobulin heavy and light chains. **B** Isoelectric focusing of salt-soluble proteins from mature barley grain. *Track j*, Pirrka; *k*, Bomi. The gel is stained for amylolytic activity. α-Amylase activity is not present, and the major β-amylase bands in the two lines are indicated by *solid arrows*. A minor β-amylase band in Bomi is indicated by a *broken arrow*

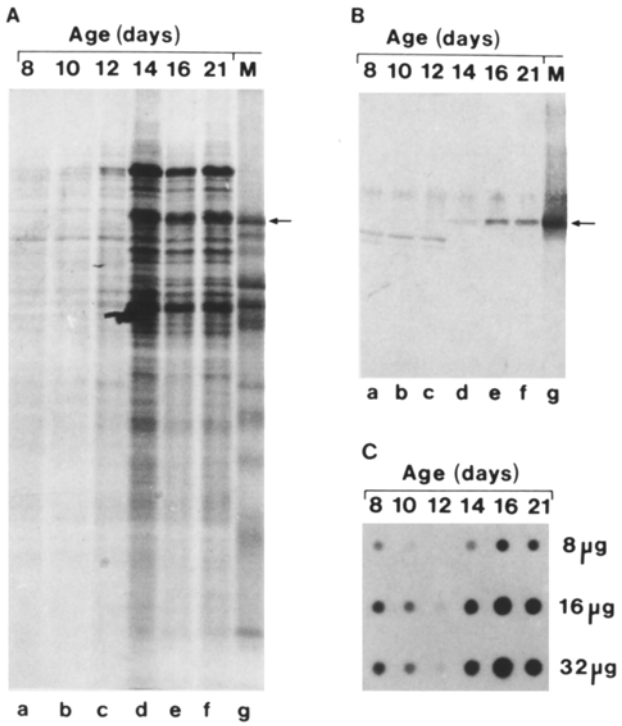

Fig. 2 A–C. β-amylase protein and mRNA in developing seeds of barley cv. Sundance **A** Analysis of total salt-soluble proteins by SDS-PAGE. *Tracks a, b* and *c* are from whole caryopses at 8, 10 and 12 d after anthesis, respectively; *d, e* and *f* are from isolated endosperms at 14, 16 and 21 d after anthesis, respectively; *g* is from mature grain. The *arrow* in *g* indicates the major β-amylase band present in mature grain. **B** Western-blot analysis of the samples shown in **A**, using β-amylase antiserum. The *arrow* in *g* indicates the major β-amylase band present in mature grain. **C** Hybrid-dot analysis of the abundance of β-amylase mRNAs. Glyoxal-denatured total polysomal RNA was extracted from whole caryopses at 8, 10 and 12 d after anthesis, and isolated endosperms at 14, 16 and 21 d after anthesis. 8, 16 and 32 µg were dotted onto "Gene Screen Plus" and probed with the ^{32}P-labelled *Acc*1 fragment of the β-amylase cDNA clone pcβC51 (Kreis et al. 1987)

ent in the same relative amounts in the protein fractions extracted without (free β-amylase) and with a reducing agent (bound β-amylase) (not shown).

Although there is genotypic variation in the relative amount of the M_r-60000 band (more being present in Hiproly and Risø 56, and less in Risø 1508), its mobility is constant (Fig. 1 A).

Analysis of β-amylase isoenzymes by isoelectric focusing showed more complex patterns, with two different types present in the eight lines. The first pattern, present in Vada, Pirrka and Sundance, consisted of seven major bands with pIs ranging from about 5.0 to 5.7 (Fig. 1 B, track j). The second pattern, present in Hiproly, Bomi, Carlsberg II, Risø 1508 and Risø 56, consisted of nine major and one minor band, with pIs between about 5.0 and 6.0 (Fig. 1 B, track k). Risø 1508 contained lower amounts of all the bands than did Bomi (not shown).

Synthesis of β-amylase in developing barley endosperms. Whole developing caryopses of cv. Sundance were prepared at 8, 10 and 12 d after anthesis, and endosperms at 14, 16 and 21 d after anthesis. Analyses by SDS-PAGE and Western-blot of salt-soluble protein fractions extracted from these samples are shown in Fig. 2, parts A and B respectively. The major form of β-amylase present in mature grain (M_r 60000) was only found after 14 d after anthesis, but two lower-M_r bands were present at earlier stages of development (8, 10, 12 d). The diffuse higher-M_r band was present at all ages. To confirm that the low-M_r immature forms were not derived from the M_r-60000 form by proteolytic digestion (in vivo or during preparation), extracts from 10-d-old caryopses and 16-d-old endosperms were mixed and incubated at 20° C for up to 18 h. No changes in the proportions of the bands were observed (not shown).

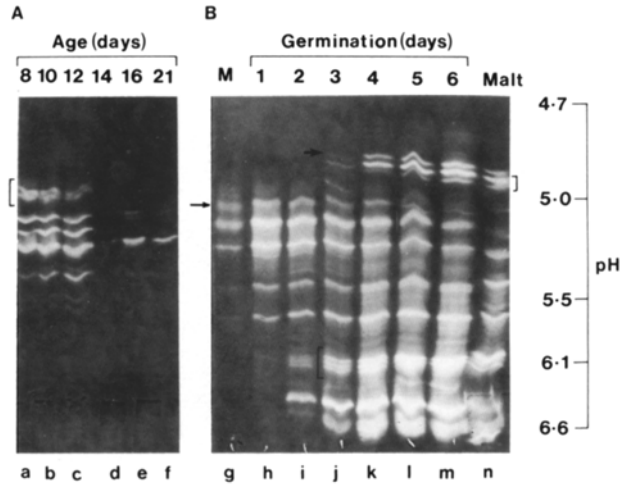

Fig. 3A, B. Isoelectric focusing of salt-soluble proteins from developing grain of barley cv. Sundance (**A**) and from mature and germinating grain of barley cv. Sundance, and malted grain of cv. Maris Otter (**B**). The gel is stained for amylolytic activity. *Tracks a, b* and *c* are from whole caryopses at 8, 10 and 12 d after anthesis, respectively; *d, e* and *f* are isolated endosperms at 14, 16 and 21 d after anthesis, respectively; *g* is mature grain; *h–m* are germinating grain at 1, 2, 3, 4, 5 and 6 d, respectively (all cv. Sundance) and *n* is commercial malted grain (cv. Maris Otter). The "green" *α-Amy2* bands are *bracketed* in *track a,* and the "malt" *α-Amy1* bands in *track j.* The other bands correspond to *β*-amylase isoenzymes. The *arrow* in *track j* indicates a high-pI *β*-amylase isoenzyme that appears during germination. The *arrow* in *track g* indicates a major isoenzyme present in the mature grain but not the 21-d-old endosperms

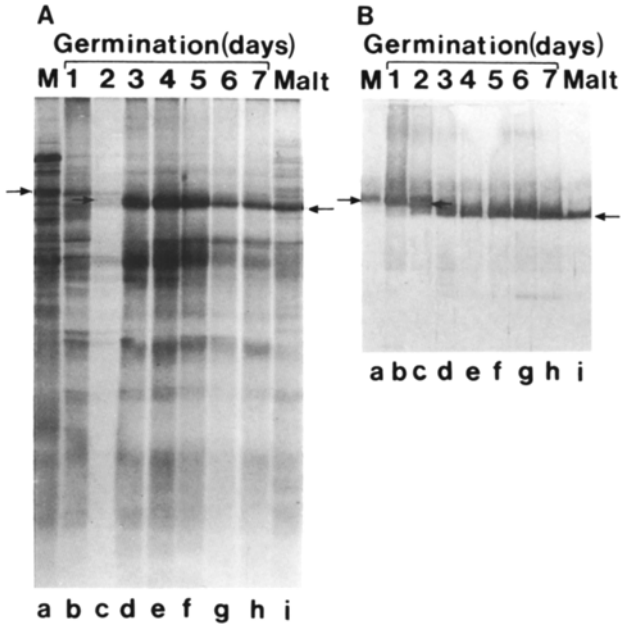

Fig. 4A, B. *β*-Amylase in germinating and malted seeds of barley cv. Sundance. **A** Analysis by SDS-PAGE of total salt-soluble proteins from mature and germinating barley grain of cv. Sundance and malted grain of cv. Maris Otter. *Track a,* mature grain; *b–h,* germinating grain at 1, 2, 3, 4, 5, 6 and 7 d, respectively (all cv. Sundance); *i,* malted grain (cv. Maris Otter). The major grain and malt forms of *β*-amylase are indicated by *arrows* in *tracks a* and *i* respectively. The *arrow* in *track c* indicates an intermediate form of the enzyme present in germinating grain. **B** Western-blot analysis of the samples shown in **A** using *β*-amylase antiserum. The major grain and malt forms of *β*-amylase are indicated by *arrows* in tracks *a* and *i*, respectively. The *arrow* in *track c* indicates an intermediate form of the enzyme present in germinating grain

The pattern of isoenzymes also changed between 12 and 14 d (Fig. 3A, tracks a–f), with no major bands in common between the two patterns. The 8- 10- and 12-d samples had "green" α-amylase bands (bracketed in Fig. 3, track a) controlled by the *α-Amy2* locus. In contrast to the SDS-PAGE analyses, differences were present between the patterns of isoenzymes present in the 14- to 21-d-old endosperms (Fig. 3A, tracks d-f) and in the mature whole grain (Fig. 3B, track g). The most acidic band in the 14- to 21-d-old endosperms was more intense in the mature grain, and a further major band of even lower pI was also present. The latter band is arrowed in Fig. 3B, track g.

Determination of the amount of *β*-amylase mRNA by hybrid-dot analysis (Fig. 2C) also showed differences between the earlier and later stages of development. The amount was high at 8 d, decreased at 10 and 12 d and then increased to reach a peak at 16 d. This indicates that the forms of *β*-amylase present between 8 and 12 d and between 14 and 21 d are encoded by separate populations of mRNAs, whose transcription is differentially regulated during grain development.

As a control the accumulation of B hordein, the major storage protein, and the population of mRNAs encoding it were determined in the same samples. Hordein mRNAs were first detected at 12 d, and hordein protein at 14 d. This is consistent with previous studies (Rahman et al. 1982, 1984), and indicates that the low *β*-amylase mRNA levels observed at 12 d did not result from degradation during harvesting and storage of the tissue, or during extraction of the RNAs.

β-Amylase in germinating and malted barley grains. Germination or malting resulted in loss of the M_r-60000 form of *β*-amylase and the appearance of a lower-M_r form (Fig. 4A, B). This conversion has been shown to result from proteolysis rather than from de novo synthesis, and appears to take place via a form of intermediate molecular weight, which is most clearly seen in the Western-blot analysis of the fraction from the 2-d-germinated grain (arrowed in Fig. 4B, track c). The forms present in the 7-d-germinated grain and the commercial malt

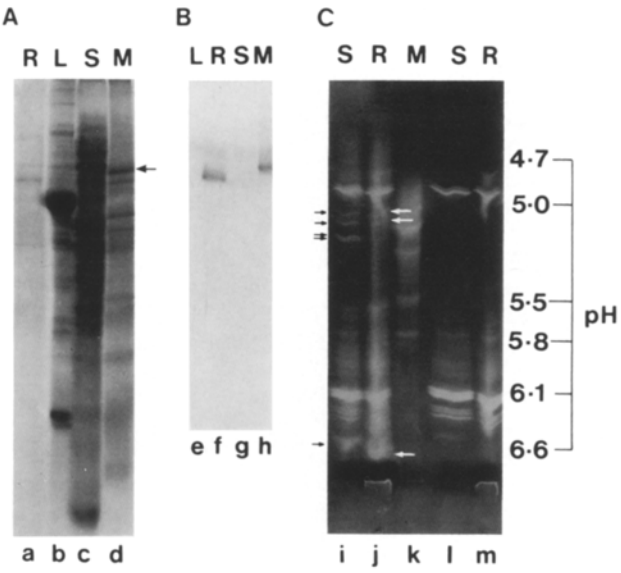

Fig. 5A–C. β-Amylase in vegetative tissues and mature seeds of barley cv. Sundance. **A** Analysis of total salt-soluble proteins by SDS-PAGE. *Track a*, roots; *b*, leaves; *c*, shoots; *d*, mature seeds. The *arrow* in *track d* indicates the major grain form of β-amylase. **B** Western-blot analysis of the samples used in **A** using β-amylase antiserum. *Track e*, leaves; *f*, roots; *g*, shoots; *h*, mature grain. **C** Isoelectric focusing of total salt-soluble proteins. The gel is stained for amylolytic activity. The samples in *tracks a, b* and *c* are untreated and show total amylolytic activity. The samples in *tracks d* and *e* have been heated at 70° C for 20 minutes to inactivate β-amylase. *Tracks i* and *l* are from shoots, *j* and *m* from roots and *k* from mature grain. The *arrows* in *tracks a* and *b* indicate the β-amylase isoenzymes identified by their lability to heating

had identical M_rs (Fig. 4A, B, tracks h, i), despite the use of two different cultivars (Sundance and Maris Otter, respectively). Similar results were observed when free and bound fractions were extracted from the germinating grain, except that the conversion to the lower-M_r form occurred more slowly in the bound fraction (not shown).

A number of new β-amylase isoenzyme bands appeared during germination (Fig. 3B, tracks h–m), with a range of pIs. They included one component with a slightly lower pI than the "green" α-amylase bands (arrowed in Fig. 3B, track j). A triplet of high-pI α-amylase bands controlled by the *α-Amy1* locus also appeared (see bracket in Fig. 3B, track j). A different pattern of β-amylase bands was present in the commerical malt (Fig. 3B, track n), presumably due to allelic variation.

β-amylase in vegetative tissues. Although β-amylase is only present in small amounts in vegetative tissues (too low to identify in the stained gel in Fig. 5A), it can be demonstrated in leaf, shoot and root tissues by Western-blot analysis (Fig. 5B). Whereas the leaf and shoot extracts contained only the diffuse high-M_r band present in all the seed extracts, the root extract also contained a low-M_r form, which co-migrated with those present in the young developing caryopses, and in the germinated and malted grains.

Because the levels of β-amylase in vegetative tissues were so low, the isoenzyme patterns on isoelectric focusing were indistinct. An extract from shoots contained a number of bands with amylolytic activity, of which several were labile on heating to 70° C and therefore presumed to correspond to β-amylase (Fig. 5C, compare tracks i and l). Four such bands had similar pIs to the most acidic bands present in the 8- to 12-d-old developing caryopses (compare Fig. 3A, tracks a–c and Fig. 5C, track i). In addition a diffuse high-pI band was also present. These bands are arrowed in Fig. 5C, track i.

Although the root extract gave even less distinct patterns than the shoot extract, it was again possible to identify β-amylase isoenzymes by comparison of non-heated and heated extracts (Fig. 5C, compare tracks j and m). The major band (arrowed in Fig. 5C, track j) had a higher pI than the isoenzymes present in the shoots, dry seeds (Fig. 5C, tracks i and k, respectively) and developing caryopses and endosperms (compare Fig. 3A), while at least two minor low-pI bands were also present (arrowed in Fig. 5C, track j).

Comparison of the heated and non-heated extracts from the two tissues indicated that most of the bands showing amylolytic activity corresponded to α-amylase.

Discussion

The present study, combining Western-blot analysis after SDS-PAGE with separation of isoenzymes by isoelectric focusing, shows multiple forms of β-amylase in the seed and vegetative tissues of barley. The forms identified by Western-blotting, which we will call isoforms to distinguish them from the isoenzymes separated by isoelectric focusing, differ in their M_rs, and are summarised in Fig. 6. A high-M_r isoform, called A, is present as a diffuse band in all tissues, and is the only form present in leaves and shoots. Isoform B is the M_r-60000 band present in mature grain, and is converted on germination or malting via isoform C to isoform D. Bands with the same M_r as form D are also present in the roots and in the young developing caryopses, and as a trace component

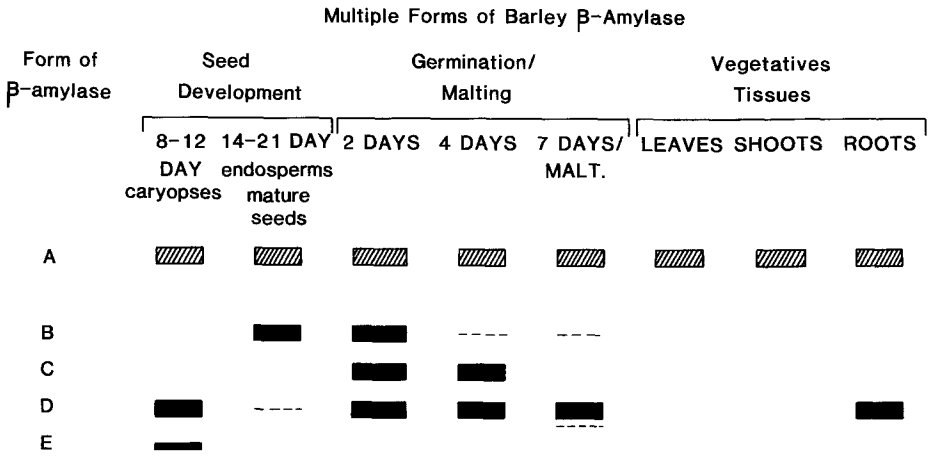

Fig. 6. Summary of the isoforms of β-amylase identified by SDS-PAGE and Western blotting of fractions from seed and vegetative tissues of barley

in the mature grain. Finally, isoform E is a relatively minor component in the young caryopses only.

Isoform B present in the mature grain is clearly the product of the *β-Amy1* locus. This locus is located on the long arm of barley chromosome 4 and has been shown to control the major grain forms of β-amylase (Powling et al. 1981; Allison and Ellis 1973; Kreis et al. 1988). Although it controls a number of isoenzyme bands separated by isoelectric focusing (see Fig. 1, part B), DNA restriction fragment analysis has shown a maximum of two gene copies per haploid genome (Kreis et al. 1988). The origins of the multiple bands observed on isoelectric focusing are not known, but post-translational processing and modification during extraction and separation may both contribute. Because such events may vary in different tissues the isoenzyme patterns must be treated with caution when considered in relation to the genetic and molecular relationships of the different isoforms.

The additional isoforms (C and D) and isoenzymes present in the germinating and malted grain were almost certainly derived from isoform B by post-translational proteolytic cleavage. Lundgard and Svensson (1986) recently reported the characterization of four forms of β-amylase from barley. Form 1 was the major form present in the mature grain, had an M_r (59700) almost identical to that predicted from the cDNA sequences (59660), and clearly corresponded to our isoform B. Forms 2, 3 and 4 had M_rs of 58000, 56000 and 54000, respectively, and resulted from limited proteolytic digestion of form 1. Although all these forms were present in the mature grain, the conversion of form 1 to form 4 was catalysed by malt protease(s), and to the intermediate forms 2 and 3 by crude malt enzymes. We assume, therefore, that our isoform D corresponds to their form 4, and our isoform C to their form 2 or 3. We do not know why we only detected one intermediate form compared with their two. Also, their three forms all had higher pIs than form 1, whereas our isoenzyme analyses showed additional bands with higher and lower pIs than those of major isoenzymes present in the mature seed. It is of interest that complex proteolytic processing also occurs in the C-terminal domain of the β-amylase of *Bacillus polymyxa*, while the mature enzyme has regions of sequence homology with the barley enzyme (Kawazu et al. 1987).

Bands with the same M_rs as isoform D were also present in the young developing caryopses and in roots, the former tissue also containing a minor isoform called E. Isoforms D and E were synthesised in developing caryopses before form B, and conversion did not occur when extracts from young caroypses and older endosperms were mixed and incubated. Also the major isoenzymes present in the young developing caryopses had pIs different from those that appeared during germination. This indicates that the isoforms of β-amylase present in the young caryopses did not result from proteolysis of isoform B. It is possible that these isoforms were derived from maternal pericarp tissue, rather than the zygotic endosperm. This is the case with the "green" *a-Amy2* isoenzymes that are present in the same tissue of wheat (Daussant and Renard 1987).

The dot-blot analysis showed two peaks of β-amylase mRNAs, which coincided with the accumulation of the early caryopsis and late endosperm isoforms respectively (i.e. isoforms D+E and isoform B). This indicates that isoforms D+E present in the young caryopses are the product of separate gene(s) to isoform B present in the mature grain (and to isoforms C and D in the germinating grain), and that these genes are differentially regulated.

The origin and relationships of the D isoform present in the roots are also not known. Since roots are likely to contain less proteases than germinating seeds and no trace of isoform B was found, it appears unlikely that isoform D was produced from isoform B by partial proteolysis. The isoenzyme pattern of roots also differed from that of the young caryopses, notably in the presence of a major band of high pI, although this could have resulted from tissue-specific modification of the same gene product.

The final isoform of β-amylase, A, was present as a diffuse band in all the tissues, and may correspond to a constitutively expressed form.

Although the *β-Amy1* locus controls the major grain form of β-amylase, a second locus was recently identified on the short arm of chromosome 2 by DNA restriction fragment analysis (Kreis et al. 1988). It probably consists of only one gene copy per haploid genome, and its product has not so far been identified. It could encode the constitutively-expressed A isoform or the tissue-specific D isoforms present in young caryopses and roots. Further studies at the chemical and genetic levels are clearly required to establish the relationships of the multiple molecular forms of β-amylase present in barley.

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