Immunological Identification of Organ Specific Proteins and Transcripts in Developing Barley Grains

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Z. Naturforsch. **48c**, 609–615 (1993); received February 10/May 10, 1993

Aleurone, Starchy Endosperm, Embryo, *in vitro* Translation, Tissue Specific Gene Expression

In vitro translated proteins from poly(A^+)RNA of immature barley starchy endosperm and embryos were immunoadsorbed with antibodies raised against proteins of aleurone layers, starchy endosperm and embryos. Four starchy endosperm and eight embryo specific transcripts were detected. In addition, several mRNAs were restricted to only two of the three tissues.

Comparing SDS-PAGE patterns of the *in vivo* protein extracts against which the antibodies were raised, four aleurone, six starchy endosperm and four embryo-specific protein bands were detectable. As for the *in vitro* translated proteins, several *in vivo* protein bands were here present in only two of the three tissues.

Of eight known barley grain proteins for which antibodies were available, only three were present in developing embryos.

Introduction

One approach for understanding the molecular basis of organ development and differentiation is the identification of organ specific proteins, transcripts and their genes. In spite of the long history of cereal grain protein characterization, only a few tissue specific proteins or transcripts from barley grains have been found. The main reason for this is that protein studies have mainly been motivated by nutritional or brewing problems [1, 2] rather than by problems in developmental biology. In the barley grain, consisting of the starchy endosperm, the surrounding aleurone layer und the embryo, the most abundant group of proteins is the prolamins (hordeins) [3, 4]. This heterogenous mixture of storage proteins is encoded by a large gene family, for which the high level of expression in the starchy endosperm is maintained by an upstream regulatory sequence element shared by many of the cereal prolamin genes, the -300 element [5, 6].

Among the nine different abundant barley grain proteins investigated by Mundy *et al.* [7] only ASI (amylase/subtilisin inhibitor) and PSI (protein synthesis inhibitor) were found to be regulated in a tissue-specific manner, *i.e.* being present only in the starchy endosperm. However, no data have been published on the accumulation of these transcripts in the barley embryo.

The main purpose of our present work is to identify potential organ-specific transcripts in the aleurone, starchy endosperm and embryo. Three different approaches were used. Firstly, SDS-PAGE patterns of *in vivo* proteins from the aleurone layer, starchy endosperm and embryo were compared in order to identify potential tissue-specific protein bands. Secondly, antibodies raised against fractionated proteins from the three grain tissues were used to immunoadsorb proteins translated *in vitro* poly(A^+)RNA of starchy endosperm and embryo. Thirdly, to complete the investigation by Mundy *et al.* [7] on the expression pattern of eight proteins in immature barley grains, *in vitro* translated proteins from embryo poly(A^+)RNA were immunoadsorbed with the same set of antibodies.

Abbreviations: DAP, days after pollination; poly(A^+)-RNA, polyadenylated RNA; cpm, counts per minute; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

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Verlag der Zeitschrift für Naturforschung, D-72072 Tübingen
0939–5075/93/0700–0609 $ 01.30/0

Materials and Methods

Plant material

Plants of *Hordeum vulgare* L. *var disticum cv.* Bomi were grown under continuous light at 15 °C for 16 h and 10 °C for 8 h. The plants were supplied weekly with a solution containing 0.02% (w/v) Superba, and 0.005% (w/v) calcium nitrate. Seed age was estimated as the number of days after pollination.

Antibodies

Polyclonal antibodies (kindly provided by Dr. Mundy, Carlsberg Research Centre, Copenhagen) were used against the following seed proteins:

(1) PSI, a "protein synthesis inhibitor" with a molecular mass of 31 kDa [7, 8], also referred to as protein K [9]. **(2)** Protein C, a chitinase of 28 kDa [9, 10]. **(3)** PAPI, a "probable amylase/protease inhibitor" of about 12 kDa [11, 12]. **(4)** β-amylase of 57 kDa [13]. **(5)** ASI, an α-amylase/subtilisin inhibitor of 21 kDa [14]. **(6)** The chymotrypsin inhibitors CI-1 and CI-2, with molecular masses of around 9 kDa [7, 15, 16]. In addition, antibodies against fraction X, a protein fraction isolated from beer which includes protein Z of about 40 kDa, were used [17, 18].

In vivo protein extraction and antibody preparation

Proteins were extracted from freeze-dried pure aleurone layers (21 DAP grains), starchy endosperm (21 DAP grains) and embryos (34 DAP grains). The tissues were isolated as described [19]. Two hundred milligrams of aleurone, 1.3 g of starchy endosperm and 300 mg of embryo tissue were ground with a pestle and mortar, defatted in butane-1-ol (10 ml per g, 2 × 30 min) and petrol ether (10 ml per g, 1 × 30 min) and dried *in vacuo*. Albumins were extracted in 5 ml of 5 mM dithiothreitol (DTT) (3 × 1 h). From the remaining pellet, globulins were extracted in 5 ml of 0.5 M NaCl (3 × 1 h). Following repelleting, hordeins were extracted from aleurone and starchy endosperm in 50% (v/v) isopropanol, according to Doll and Andersen [20].

Protein concentration in albumin and globulin extracts was measured with the Bio-Rad protein assay kit, and in the hordein extract by the method of Lowry *et al.* [21]. After concentrating by TCA-precipitation (albumins and globulins) or water-precipitation (hordeins), 125 µg of each protein fraction were dissolved in loading buffer and applied to gels for coomassie staining. SDS-PAGE was performed as described by Klemsdal *et al.* [22]. The remaining albumin and globulin extracts were freeze-dried before immunization.

To obtain antibodies against albumins and globulins from pure aleurone, starchy endosperm and embryo tissue, samples of 550 µg, 1500 µg and 1500 µg (albumins) and 220 µg, 360 µg and 750 µg (globulins), respectively, were injected 10 times subcutaneously in rabbits according to standard procedures [23].

Tissue preparation and isolation of poly(A$^+$)RNA

For isolation of poly(A$^+$)RNA, pure starchy endosperm tissue was prepared from 21 DAP grains, and embryos from 26 DAP grains. In the classification system for wheat caryopse [24], the barley embryos and endosperms are both in stage IV, characterized by increasing cell size and storage protein accumulation, *i.e.* when tissue differentiation is completed. This corresponds to the late cotyledon-early maturation phase, as defined by Galau *et al.* [25]. Twenty one days corresponds to the peak of storage protein mRNA synthesis in the starchy endosperm, and is the last stage at which the tissue easily can be separated from the surrounding aleurone cells [7, 26, 27].

Poly(A$^+$)RNA was isolated as described by Mundy *et al.* [28].

In vitro translation

Poly(A$^+$)RNA was *in vitro* translated in rabbit reticulocyte lysate supplied with [^{35}S]methionine, as described by Mundy *et al.* [7].

Analysis of in vitro synthesized proteins

TCA precipitable radioactivity was determined and aliquots taken for analysis of total *in vitro* synthesized polypeptides (0.5×10^5 cpm/lane), for immunoadsorption of protein fractions (5.0×10^5 cpm/lane), and for immunoadsorption of specific proteins (7.5 or 10×10^5 cpm/lane). Immunoadsorption was otherwise done as described by Mundy *et al.* [7].

SDS-PAGE was performed as described by Klemsdal *et al.* [22]. The ^{14}C labelled protein marker consisted of 6.5, 12.5, 25, 46 and 69 kDa

proteins (Amersham). Gels were prepared for fluorography as described by Jonassen *et al.* [29]. Films (Hyperfilm β-max) were exposed from 1 to 35 days. Densitometric scanning of fluorograms was done in a Shimadzu CS-930 spectrophotometer. Relative amounts of given single proteins were estimated as described by Mundy *et al.* [7].

Results

Purity of isolated tissues

The purity of the aleurone layer preparation is demonstrated in Fig. 1, showing that the B1-hordein, the major protein of the starchy endosperm (lane 3), is virtually absent from aleurone extracts (lane 2). The most abundant hordein of the aleurone (Cy, 66 kDa) does not correspond in molecular mass to any previously identified starchy endosperm hordein, whereas Cx (56 kDa) corresponds in molecular mass to the Cb-hordein described by Klemsdal *et al.* [22]. Cross sections of isolated starchy endosperms and embryos (not shown) showed that the preparations were free from contaminating tissues.

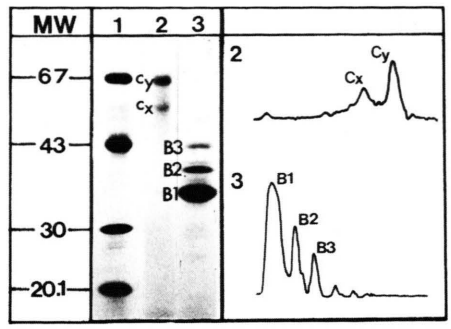

Fig. 1. Coomassie-stained SDS-polyacrylamide gel of prolamins from aleurone (lane 2) and starchy endosperm (lane 3) of 21 DAP barley grains. Curves in the right panel represent densitometric scans of lane 2 (upper part) and lane 3 (lower part). Lane 1 contains molecular mass markers (kDa, Pharmacia).

In vivo proteins

SDS-PAGE patterns of the albumin and globulin extracts of aleurone layers, starchy endosperm and embryos used for raising antibodies (see below) are shown in Fig. 2. Four aleurone- (lane 2, band 2 and lane 4, band 1, 2, 4), six starchy endosperm- (lane 3, band 1, 2, 4 and lane 5, band 1, 2, 6), and four embryo-specific (lane 7, band 1, 2, 3 and lane 8, band 1) protein bands, were identified on the gels. In addition, a 31 kDa albumin band is present in both the aleurone extract (lane 2, band 1) and the embryo extract (lane 7, band 5), but absent or highly reduced in the starchy endo-

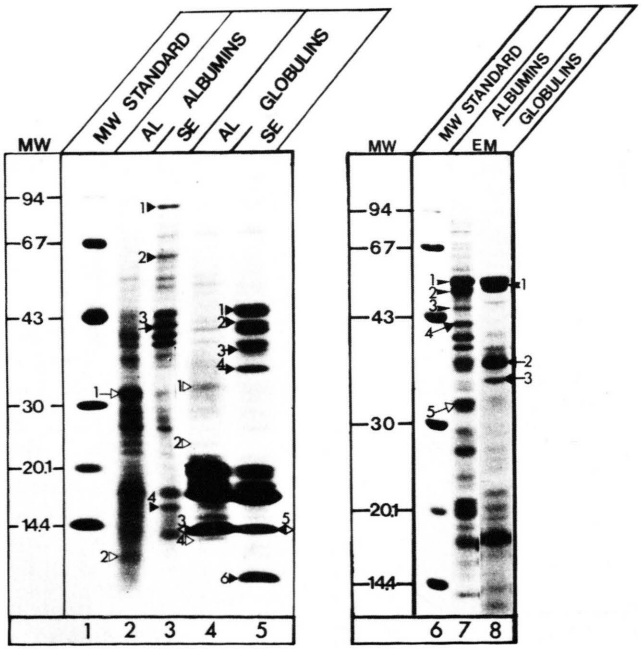

Fig. 2. Coomassie-stained SDS-polyacrylamide gel of albumins and globulins of 21 DAP aleurone (AL, lane 2 and 4), 21 DAP starchy endosperm (SE, lane 3 and 5) and 34 DAP embryos (EM, lane 7 and 8). Lane 1 and 6 contain molecular mass markers (kDa, Pharmacia). The embryo lanes were run on a separate gel, resulting in slightly different migration of the proteins from the two other tissues. Different arrows mark out protein bands specific to the embryo (►), starchy endosperm (▶), aleurone (▷), embryo and starchy endosperm (—▶), embryo and aleurone (—▷), starchy endosperm and aleurone (◗).

Table I. Molecular mass estimates, given in kDa, of barley grain tissue-specific *in vivo* and *in vitro* protein bands. For explanation, see the text.

Aleurone	*in vivo*	32^2 23^2 13^2 12^1
Starchy endosperm	*in vivo*	88^1 62^1 45^2 42^2 16^1 11^2
	in vitro	90^1 57^1 19^1 15^2
Embryo	*in vivo*	54^1 52^2 50^1 46^1
	in vitro	92^1 87^1 82^1 67^1 54^1 47^1 43^1 25^1
Starchy endosperm and aleurone	*in vivo*	14^2
	in vitro	42^1 41^2 $39^{1.3}$ $39^{2.3}$ $37^{2.3}$ 31^2 29^1 14^1 $7^{1.3}$
Embryo and aleurone	*in vivo*	31^1
	in vitro	49^2 40^1 $6^{1.3}$
Starchy endosperm and embryo	*in vivo*	42^1 38^2 35^2
	in vitro	52^1 32^2 27^2

[1] Albumin.
[2] Globulin.
[3] Detected only by antibodies raised against aleurone proteins.

sperm extract (lane 3). A 14 kDa globulin band is present in the aleurone and starchy endosperm extracts (lane 4, band 3 and lane 5, band 5), but not in the embryo (lane 8). In addition, a 42 kDa albumin band, a 38 kDa and a 35 kDa globulin band were present in the starchy endosperm (lane 3, band 3, and lane 5, band 3 and 4, respectively) and the embryo (lane 7, band 4, and lane 8, band 2 and 3, respectively), but absent from the aleurone extracts (lanes 2 and 4). Estimated molecular mass and solubility characteristics of these protein bands are listed in Table I.

In vitro proteins

Polyspecific antibodies: The fluorograms of the SDS-PAGE pattern of total *in vitro* translated proteins from poly(A$^+$)RNA of starchy endosperm and embryos are shown in Fig. 3, lanes 3 to 16. Comparison between the *in vivo* proteins and *in vi-*

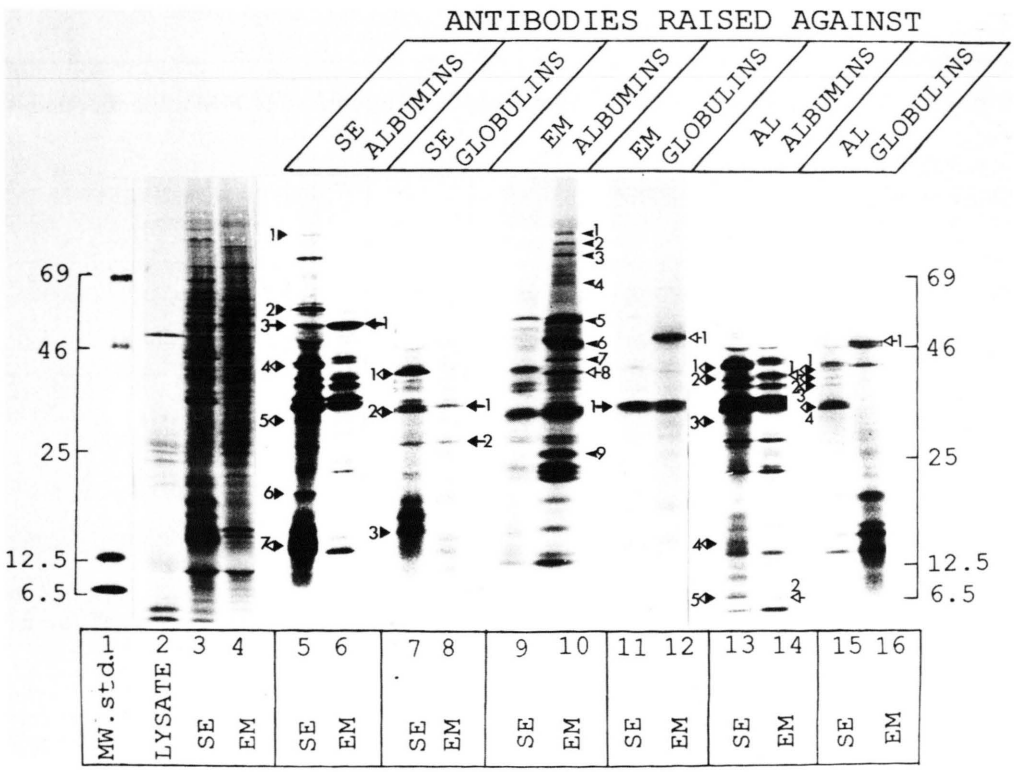

Fig. 3. Fluorogram of SDS-polyacrylamide gels of [^{35}S]methionine-labelled polypeptides synthesized *in vitro* from mRNA of 21 DAP starchy endosperm (SE) and 26 DAP embryos (EM), adsorbed with antibodies directed against protein fractions as indicated in the top panel (lanes 5–16). Lane 1 contains the molecular mass markers (kDa), lane 2 shows the rabbit reticulocyte translation lysate preparation without addition of mRNA, and lanes 3 and 4 contain the total translation products from the starchy endosperm and embryo, respectively. For symbols, see legend of Fig. 2.

tro proteins adsorbed with antibodies against the respective protein fractions (Figs. 2 and 3), indicate a good immunogenic response to the extracted proteins.

Using antibodies directed against starchy endosperm albumins and globulins to immunoadsorb proteins from starchy endosperm poly(A^+)RNA, six albumins (lane 5, bands 1, 2, 4, 5, 6, 7) and three globulins (lane 7, bands 1, 2, 3) were adsorbed which were not detectable in the embryo translation products using the same antibodies (lanes 6 and 8). Three of these albumin bands (lane 5, bands 4, 5, 7) and two of the globulin bands (lane 7, bands 1, 2) were also adsorbed using antibodies directed against aleurone proteins (lane 13, bands 1, 3, 4, and lane 15, bands 1, 4, respectively).

Using antibodies directed against protein extracts from embryos to adsorb proteins translated *in vitro* from embryo poly(A^+)RNA, 9 albumins (lane 10, bands 1–9) and one globulin (lane 12, band 1) were detectable that were absent from the starchy endosperm translation products (lane 9 and 11). Two of these protein bands (lane 10, band 8 and lane 12, band 1) were detectable when antibodies directed against aleurone proteins were used to adsorb the same *in vitro* translated proteins (lane 14, band 1 and lane 16, band 1). The protein bands are listed in Table I.

By immunoadsorption of *in vitro* translated proteins from embryo and starchy endosperm tissues with antibodies against aleurone proteins, a further search for transcripts present in only two of the three grain tissues was performed. This approach identified nine protein bands common to both the aleurone and the starchy endosperm (lane 13, bands 1–5, and lane 15, bands 1–4), but absent from the embryo (lanes 14 and 16). Three protein bands common to the aleurone and the embryo (lane 14, band 1, 2, and lane 16, band 1), but absent from the starchy endosperm (lanes 13 and 15) were also detected. In addition, some protein bands were detected in both embryo and starchy endosperm *in vitro* translation products only with antibodies raised against proteins from embryo and starchy endosperm tissue, indicating absence of these proteins from the aleurone cells (lane 6, band 1; lane 8, bands 1, 2; and lane 11, band 1). Estimated molecular masses of the different classes of tissue specific protein bands are listed in Table I.

Monospecific antibodies: Among the eight proteins assayed for in the present experiment, PSI, protein C and PAPI were not detectable in either the starchy endosperm or the embryo *in vitro* translation products. Among the remaining proteins, ASI and protein Z (in fraction X) were only detected in the starchy endosperm *in vitro* translation products, whereas β-amylase, CI-1 and CI-2 were also present in the embryo, although β-amylase and CI-1 were reduced 100-fold relative to the starchy endosperm level (Fig. 4 and Table II). The quantity of CI-2 in the embryo is comparable to that in the starchy endosperm. However, the molecular mass of CI-2 is lower in

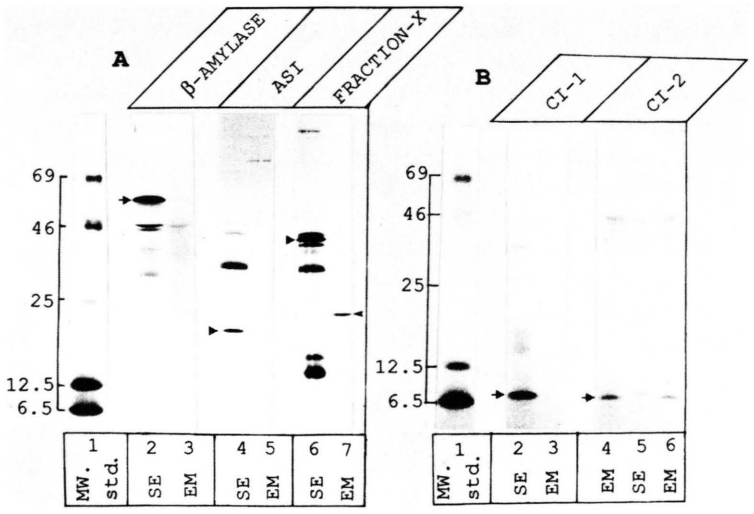

Fig. 4. Fluorogram of SDS-polyacrylamide gel of [^{35}S]methionine labelled polypeptides synthesized *in vitro* from mRNA of 21 DAP starchy endosperm (SE) and 26 DAP embryos (EM), adsorbed with antibodies as indicated in the top panel, A) β-amylase, ASI, Fraction X, B) CI-1 and CI-2. Molecular weights are in kDa. The electrophoresis of CI-1 and CI-2 was run on a 11–22.4% gradient gel instead of a 8–18% gradient gel, to improve the separation of the two MW-forms of CI-2 in EM and SE. More protein was applied in lane 4 than in lanes 5 and 6. For symbols, see legend of Fig. 2.

Table II. Relative amounts of specific polypeptides immunoadsorbed from starchy endosperm and embryo *in vitro* translation products, estimated as described by Mundy *et al.* [7]. Densitometric scanning of fluorographs in a Shimadzu CS-930 scanner was used to estimate the relative amounts of radioactivity in lanes.

Protein	MW [kDa]	Number of methionines	Starchy endosperm	Embryo
ASI	21	2	149	–
Protein Z	40	4	141	–
β-Amylase	57	15	21	0.3
CI-1	9	2	58	0.5
CI-2	9	1	14	26

the embryo than in the starchy endosperm, indicating the expression of a different gene in this tissue. The fraction X antibodies adsorb a 23 kDa protein band in the embryo that is absent from the starchy endosperm (Fig. 4A, lane 6). This protein is not adsorbed from aleurone *in vitro* translation products (Cathy Kalvenes, personal commun.), and is thus a potential embryo-specific protein.

Discussion

Our previous work has focused on the identification of aleurone-specific transcripts and the identification of promoter elements specifying gene transcription in the aleurone layer [19, 30]. During the work with the cDNAs representing aleurone-specific transcripts as well as transcripts common to both the aleurone and the embryo, a need for an overview of tissue-specific proteins and transcripts in the barley grain was felt. In the present study, 14 potentially tissue-specific *in vivo* albumin and globulin protein bands were identified. In this paper, we present SDS-PAGE profiles of proteins extracted from pure developing aleurone layers. A striking feature of these gels is the lack of abundant aleurone-specific protein bands. This observation is compatible with the lack in aleurone cells of massive protein stores like the highly abundant B1 hordein of the protein bodies in the starchy endosperm [31, 32]. One possible candidate for the unidentified protein store in the aleurone grains [33, 34] is the prolamin Cy (Fig. 1). The lack of highly abundant aleurone-specific gene-products also agrees well with the observation that the aleurone-specific transcripts identified by differential screening of a 20 DAP aleurone cDNA library are of moderate-to-low abundance [19].

Based on Northern analysis of RNA from aleurone layers, these transcripts have the capacity for encoding proteins in the molecular range of 10 to 20 kDa. Thus, among the cDNAs representing aleurone-specific transcripts some might encode the aleurone-specific *in vivo* proteins contained in the 12 und 13 kDa bands (Table I). No clones corresponding to the potentially aleurone-specific 23 and 32 kDa protein bands (Table I) were detected in the screening for aleurone specific cDNAs. One possible explanation for this is that the transcripts accumulate at an earlier stage than 20 DAP and therefore have escaped our detection. One such early transcript has been identified in the barley embryo by Bartels *et al.* [35].

Based on the immunological properties of *in vitro* translated proteins adsorbed with antibodies directed toward different protein fractions, four starchy endosperm and eight embryo-specific transcripts were identified in the present experiments (Table I). Comparison of the *in vitro* translated proteins with published data and with *in vivo* proteins is difficult for several reasons. Molecular masses of *in vitro* translation products may be higher than those of their corresponding *in vivo* proteins due to unprocessed leader sequences, and protein band intensities on fluorograms depend on the number of methionine residues, which is highly variable (Table II).

In spite of these complications, several tissue-specific bands with similar molecular masses do correspond to each other on the *in vitro* and *in vivo* gels. Thus, in the starchy endosperm, the *in vitro* and *in vivo* albumin bands of 90 and 88, 57 and 62, 19 and 16 as well as the 15 and 11 kDa globulin bands, respectively (Table I), may represent the same proteins. The same may also be true for the three embryo-specific albumins with molecular masses of 54, 47 and 43 kDa *(in vitro)* and 54, 50 and 46 kDa *(in vivo)*, respectively.

In addition to the tissue-specific protein bands, a number of bands were identified in two of the three grain tissues (Table I). Such gene products may reflect common functions shared by the two tissues in question. One example of a common function between two barley grain tissues is the secretion of hydrolytic and proteolytic enzymes by the scutellum of the embryo and by the aleurone layer during germination [28, 33, 34, 36]. Whether this particular functional similarity is also reflected

in the expression of identical genes during grain development is, however, unknown.

Taking also quantitative aspects into consideration, the present investigation reveals large differences in the overall SDS-PAGE patterns of *in vitro* translated proteins from the three different grain tissues. However, we have listed as tissue-specific those *in vitro* translated proteins that could be identified in only one of the three tissues.

Having identified tissue-specific transcripts the question arises how they can be isolated for use in developmental biology studies. One method to isolate the transcripts is differential screening of cDNA libraries made from poly(A^+)RNA of the tissue and stage under investigation [19]. Immunoscreening of selected cDNA clones in expression libraries, using the same set of antibodies as used in the present investigation, can also be carried out for the identification of cDNAs encoding the tissue-specific proteins described in this paper.

Acknowledgements

We thank Dr. Odd-Arne Olsen for guidance, Dr. Adrian Smith at The Norwegian College of Veterinary Medicine, for help and guidance in producing the polyspecific antibodies, and Dr. John Mundy at the Carlsberg Research Center, Department of Biotechnology, for providing the monospecific antibodies. We are also grateful to Solveig Hauge-Engebretsen for excellent technical assistance, and to the Phytotron at the University of Oslo, for the use of their facilities. The work was funded by the Norwegian Agricultural Research Council (NLVF).

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