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CHANGES IN PHOSPHORYLATED AND UNPHOSPHORYLATED NUCLEAR PROTEINS DURING GERMINATION IN BARLEY

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Key Word Index–*Hordeum vulgare*; Gramineae; barley; nuclear proteins; gel electrophoresis; germination; phosphorylation of protein.

Abstract—Nuclear proteins from barley have been separated by two-dimensional gel electrophoresis. Changes in the patterns of phosphorylated and unphosphorylated nuclear proteins which accompany germination have been examined. Only about half the nuclear proteins present in ungerminated embryos are still detectable after 24 hr germination.

INTRODUCTION

In a previous paper we reported that the nuclear phosphorylated protein profile appeared to be altered during barley embryo germination [1]. The method used in that paper for protein separation was single-dimension gel electrophoresis. The limitations of separative power in this method and the apparent complexity of phosphorylated nuclear proteins in barley failed to provide a reasonable answer to either of two questions. Firstly, how many phosphorylated nuclear proteins are there in barley and secondly are there qualitative changes in the phosphorylated nuclear proteins accompanying germination? This paper reports an examination of changes in phosphorylated nuclear proteins by a much more discriminating two-D gel electrophoretic method. As a consequence, reasonable answers can now be offered to both questions. In addition qualitative changes in unphosphorylated nuclear proteins during germination are also reported.

RESULTS

In experiments on the protein composition of nuclei and its variation during development it is necessary to ensure that a representative population of nuclei be isolated, otherwise changes may be the result of differential extraction. In an attempt to obviate this difficulty in barley estimates of nuclear yield have been made at various stages of purification. Ungerminated barley embryos contained 2·3 mg DNA/g fr. wt (average of 3 experiments). The yield in the first nuclear pellet varied between 1·6 mg and 2·07 mg DNA/g fr. wt (average 1·83 from 12 experiments). Approximately 85% of these nuclei were found to sediment in 3 min at 275 *g* with the remaining 15% sedimenting during the remaining 3 min of the first spin. The further purification described here which uses metrizamide eliminates detectable starch and gives final yields of nuclei varying from 0·9 to 1·35 mg DNA/g fr. wt (results of 4 experiments). The yields from embryos germinated for 24 hr were in the above ranges but lower on a percentage basis because the DNA content of the embryos was 2·62 mg DNA/g fr. wt. Protein–DNA ratios of the purified nuclei were 2·81 for ungerminated embryos and 3·03 for germinated embryos both values being averages of 3 experiments. Gel electrophoresis of the nucleic acids in the first nuclear pellet showed it to contain more than 95% DNA. Microscopic examination of final nuclear pellet after metrizamide purification showed it to contain many nuclei and occasional cell wall fragments. Despite extensive experimentation we were unable to find a density of metrizamide which would conveniently separate nuclei and cell wall material in ungerminated embryos without unacceptable losses of nuclei. This problem was in part caused by the apparent very wide range of densities of nuclei from ungerminated embryos.

Proteins were solubilised from nuclei using 8M urea as described by Gronow and Griffiths [2]. About 65% of the total nuclear protein soluble in 6M urea–3M NaCl can be solubilised by a prior extraction with 8M urea, in agreement with Gronow and Griffiths [2]. Gel electrophoresis of the 8M urea extract at pH 3 [1] indicated an almost total absence of histone material.

For 2-dimensional work the proteins were first separated by isoelectric focusing in a cylindrical 4% acrylamide gel using ampholine pH 3·5–10. After the electrophoresis was concluded the pH gradient was determined on control gels. The cylindrical gel was then horizontally embedded in a 9% acrylamide slab gel and separated by SLS gel electrophoresis in the vertical direction. A MW scale for the 2nd dimension was constructed by the use of marker proteins of known MW. These were loaded in slots at the sides of the cylindrical gel and electrophoresed into the slab gel at the same time. After staining and destaining, some 50–60 protein spots could be detected with loadings between 300–500 μg protein. More spots can be detected with heavier loadings.

Figure 1a shows a 2D electrophoretogram of nuclear proteins from ungerminated embryos with pH and MW

scales included. The majority of 8M soluble nuclear proteins have isoelectric points between pH's 4–6 and MWs above 40000 as in animal systems [3]. Figure 1b shows 8M urea-soluble nuclear proteins extracted from 24 hr germinated embryos. Although substantially the same number of proteins can be detected which again are mainly acidic in character, the pattern is very different between the 2 stages of germination. To enable comparisons to be made a map has been drawn in Fig. 1c showing which proteins appear to be present in both stages of germination. Only about half the proteins detected in ungerminated embryos are still detectable after 24 hr. The largest quantitative difference is seen in the protein marked by the arrow in Fig. 1a. As judged by staining

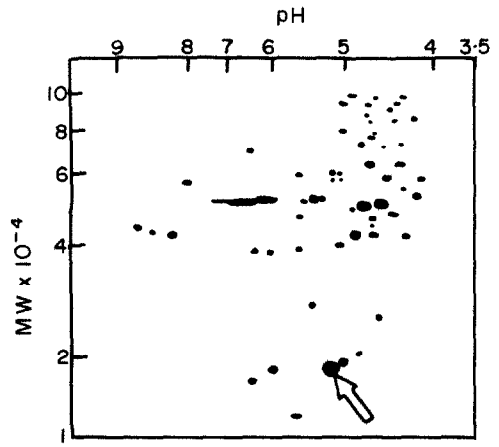

Fig. 1(a).

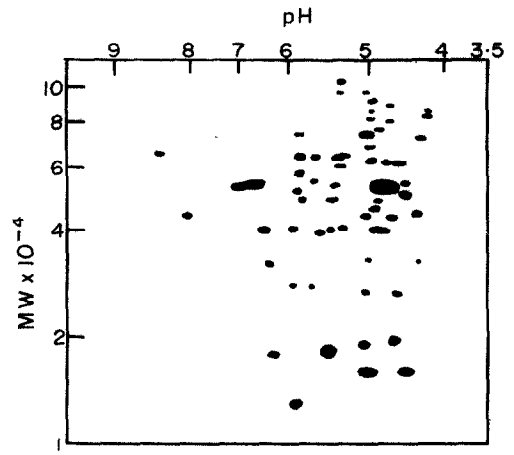

Fig. 1(b).

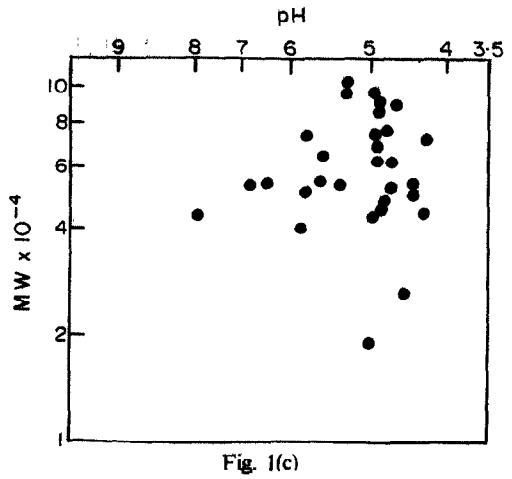

Fig. 1(c)

Fig. 1. Two-D gel electrophoretic patterns of nuclear proteins from ungerminated (1a) and germinated (1b) embryos. 1c shows positions of proteins in both ungerminated and germinated embryos.

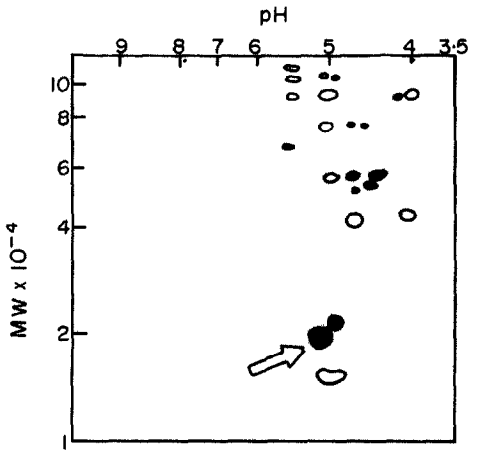

Fig. 2(a).

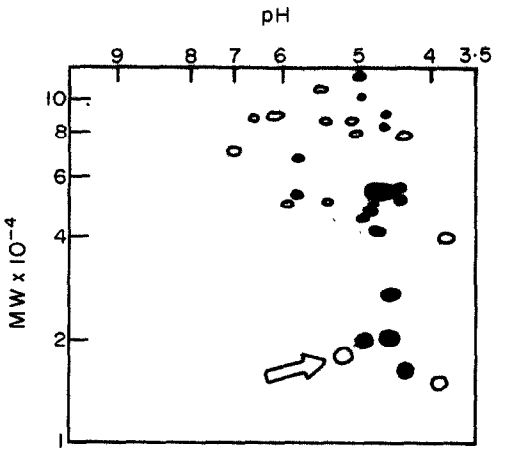

Fig. 2(b).

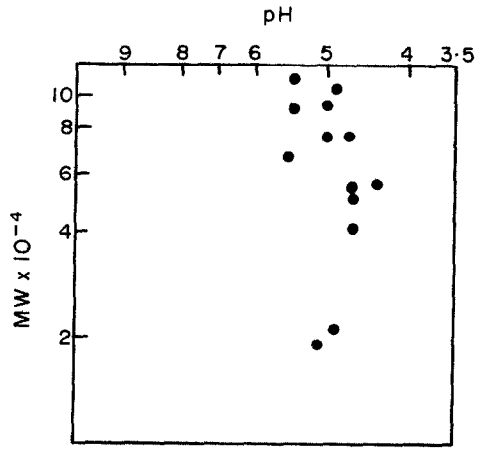

Fig. 2(c).

Fig. 2. Two-D gel electrophoretic patterns of phosphorylated nuclear proteins from ungerminated (2a) and germinated (2b) embryos. 2c shows positions of proteins present at both stages.

this protein is present in the largest amount in ungerminated embryos but is no longer detectable after 24 hr germination.

The nuclear proteins have also been phosphorylated using the nuclear protein kinase and γ-^{32}P-ATP as previously described [1]. After extraction and 2D separation autoradiographs of the gels were prepared. Figure 2a shows the 2D autoradiogram of labelled proteins in ungerminated and Fig. 2b the 2D autoradiogram of labelled proteins in 24 hr germinated embryos. Labelled spots which correspond to a stained protein have been drawn as filled spots but labelled spots which do not coincide with a stained protein have been left as open spots. The majority of phosphorylated nuclear proteins are, like the other nuclear proteins, acidic in character and have MWs above 40000. The autoradiographs provide minimal estimates of the number of phosphorylated nuclear proteins in barley, being 22 in ungerminated embryos and 30 in germinated embryos, figures not dissimilar to those found in animal cells [3]. The spot indicated by the arrow in figure 2a corresponds to the protein indicated by the arrow in Fig. 1a. It accounts for about 25% of the total radioactivity on the autoradiogram in Fig. 2a but this is reduced over 10-fold after 24 hr germination. Comparison of Figs. 2a and 2b indicates substantial changes in the pattern of phosphorylated proteins during germination and to clarify this a map has again been drawn in Fig. 2c showing the phosphorylated proteins present in both germination stages. Only just over half of the phosphorylated proteins present in nuclei of ungerminated embryos are still detectable after 24 hr.

In order to check that substantial numbers of phosphorylated proteins were not being lost in the 8M urea-insoluble protein fraction of ungerminated embryos the residue after 8M urea extraction was suspended in 6M urea/3M NaCl and the DNA centrifuged down overnight. After dialysis against 8M urea and subsequent concentration the proteins were separated two-dimensionally.

The results of staining and autoradiography are shown in Figs. 3a, b respectively. Despite extensive overloading (a mg of protein was loaded on the gels) traces of radioactivity were found in only 1 spot. Perhaps more interesting is that this 8M urea-insoluble fraction contains only a limited number of basic proteins (as well as histones) but no trace of acidic proteins.

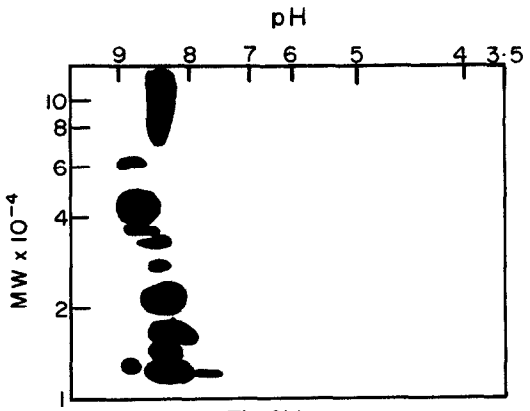

Fig. 3(a).

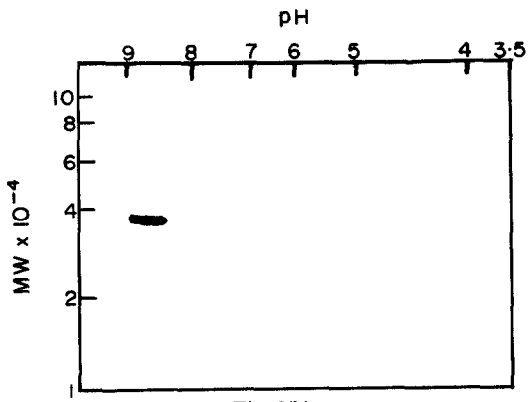

Fig. 3(b).

Fig. 3. Two-dimensional gel electrophoretic pattern of the nuclear proteins insoluble in 8M urea: a stained spots; b pattern of radioactivity.

DISCUSSION

These present results are in good agreement with those published earlier by us but using inferior and less discriminating techniques [1]. There we were able to report that germination resulted in a marked decline of at least one low MW phosphorylated protein. It is possible that this may be identical to the protein shown by the arrow in Figs. 1a and 2a. The improved separation has also indicated that the germination changes in nuclear phosphorylated protein are more complex than could be surmised from single dimension gels. The significance of these results must await further study but it can be pointed out that 24 hr germinated embryos have already commenced DNA synthesis and thus some of these changes may be related to the onset of cell division.

EXPERIMENTAL

Isolation of nuclei. Sterile barley embryos (var. Proctor) were obtained and germinated as previously described [1]. Nuclei were isolated from about 1 g batches of embryos by grinding in a Potter homogeniser (total clearance of 16/1000″) driven at 200 rpm and at 0°. The homogeniser medium was 0·3M sucrose, 0·05M Tris pH 7·4 0·01M $MgCl_2$. The homogenate was filtered through a flat bed of 2 layers of MIRA cloth, about 50 cm^2 in area, which was pre-saturated with grinding buffer. The MIRA cloth was washed with 2 vols homogenisation medium and the residue washed into and ground in a Potter homogeniser (6/1000″ total clearance) again at 200 rpm and at 0°. This homogenate was filtered as above, the two filtrates were combined and made 1% in Triton-X-100. The filtrate was centrifuged at 275 *g* for 6 min. The pellet was resuspended in 8 ml 55% w/v metrizamide [5] (Nyegaard & Co., A/S Oslo, Norway) in 0·3M sucrose, 0·05M Tris pH 7·4, 0·01M $MgCl_2$, layered over 2 ml of the 55% metrizamide and spun at 275 *g* for 6 min in a swing out rotor. The pellet consisting of starch was discarded. The supernatant was diluted with 25 ml homogenisation medium, layered over 10 ml 1M sucrose, 0·05M Tris pH 7·4 0·01M $MgCl_2$ and spun at 1000 *g* for 6 min. The pellet was resuspended in homogenisation medium and re-centrifuged at 275 *g* for 6 min. Proteins were labelled by incubating nuclei in γ-^{32}P-ATP as previously described [1].

Extraction and 2D separation of nuclear proteins. Nuclear acidic proteins were solubilised using 8M urea and maleylated as described by Gronow and Griffiths [2]. Isoelectric focussing was carried out essentially as described [2] except that the final acrylamide concentration was 4%, the gels were 8 cm in length and of a total vol of 1·5 ml. Separation was conducted at 150 V at 9° for 18 hr. The protein load for Figs

1 and 2 was 400 μg and 980 μg for Fig. 3. After the run pH gradients were determined on blank gels as described by Barrett and Gould [4] The first dimension gels were re-equilibrated to sodium lauryl sulphate as described by McGillivray and Rickwood [3] and embedded in the upper stacking gel of their SLS system. The apparatus used for slab gel electrophoresis was similar to that described by Mets and Bogorad [6]. The bottom separation gel was about 10 cm in length and had a total acrylamide content of 9%. Slots were formed in the upper stacking gel adjacent to the cylindrical gel and molecular weight marker proteins together with bromophenol blue pipetted into the slots [3]. The second dimension electrophoresis was run at 37 V and at 9° until the bromophenol blue marker reached the bottom of the gel (generally 18 hr). Gels were stained and destained as described [3], sealed in plastic bags and placed against X-ray film for autoradiography. DNA [7] and protein [8] were estimated by standard methods.

For separations shown in Fig. 1a–c, nuclei were prepared from 0 and 24 hr germinated barley embryos and the protein solubilised with 8M urea. Four hundred micrograms of protein from both embryo stages were subjected to isoelectric focussing in a cylindrical gel and pH gradients determined on blank gels. The cylindrical gels were then embedded in a slab gel and separated by SLS gel electrophoresis in the 2nd dimension. Molecular wt gradients were determined by running bovine serum albumin, ovalbumin, trypsin and cytochrome *c* in slots by the side of the cylindrical gel. The slab gels were stained with coomassie blue, destained and sealed in plastic bags.

For separations shown in Fig. 2a–c, nuclear proteins were labelled by incubating nuclei in γ-^{32}P-ATP. They were solubilised in 8M urea and 400 μg protein separated 2-dimensionally using iso-electric focussing and SLS gel electrophoresis. After staining the gels were sealed in plastic bags and autoradiographs prepared. The autoradiographs were drawn onto tracing paper and matched with the stained gel. Filled spots indicate coincidence of radioactivity with stained protein spots. Open circles indicate radioactivity which does not coincide with stained protein.

For separations shown in Fig. 3a,b, nuclei were prepared from ungerminated embryos and incubated in γ-^{32}P-ATP. The nuclei were extracted with 8M urea and the residue suspended in 6M urea–3M NaCl. The DNA was centrifuged down at 10^5g for 16 hr and the supernatant dialysed against 8M urea. The proteins (980 μg) were subjected to 2D gel electrophoresis as under Fig. 1 and autoradiographs prepared.

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