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Heterogeneity of the amino acid sequence of phytochrome from etiolated oat seedlings

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Phytochrome from etiolated oat seedlings was digested with subtilisin. A 16 kDa fragment was isolated and investigated by microsequencing. Its amino-terminal sequence SLPGGSMEV/ML revealed a heterogeneity (valine and methionine) at position 9. This proves expression of several phytochrome genes on the protein level. One can conclude from the ratio valine/methionine that the isophytochrome derived from AP5 (containing methionine) is a major gene product besides isophytochromes derived from genes AP4 and AP3 (containing valine).

Phytochrome isoprotein; Proteolysis; Microsequencing; Subtilisin nagarse; (*Avena sativa* L.)

1. INTRODUCTION

Phytochrome is the main photoreceptor for light-dependent differentiation and development of higher plants. Since the characteristics of phytochrome-mediated responses can be quite different from each other indicating more than one primary molecular mechanism [1], heterogeneity has been postulated either for the presumed phytochrome receptor(s) [2] or for phytochrome itself [3]. The restriction map polymorphism among the cDNA clones for phytochrome from etiolated oat seedlings indicated that at least four different phytochrome genes might be expressed in oat [4]. Two cDNA sequences (AP3 and AP4) were presented in full length for phytochrome and a third (AP5) in part. The authors conclude that localized changes in the protein structure deduced from the cDNA sequence could eventually contribute to functional heterogeneity in the phytochrome population although the corresponding heterogeneity has not yet been detected experimentally at the protein level. In the course of our studies on amino acid sequences of proteolytic fragments of phytochrome from etiolated oat [5], we detected a heterogeneity of the amino acid sequence which will be described in the following.

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2. MATERIALS AND METHODS

Native phytochrome (124 kDa) was isolated from 3.5-day-old etiolated oat seedlings (*Avena sativa* L. cv Pirol, Baywa München) as described [6]. The purity of the product was checked by absorption spectroscopy ($A_{667}/A_{280} = 0.99$ for the Pr form) and by SDS gel electrophoresis according to Laemmli [7]. Contaminating protein bands were not detected even with the sensitive silver nitrate stain; the limit of detection is $\leq 1\%$ of the amount of phytochrome protein.

For proteolytic degradation, phytochrome samples (500–700 μg protein) in 10 mM phosphate buffer, pH 7.8, containing 5 mM EDTA and 5% glycerol, were incubated with subtilisin nagarse [Sigma, from *B. subtilis*, 16 U/mg, 1% (w/w) based on phytochrome] in the dark at 20°C for 24 h. The reaction was then stopped by addition of

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phenylmethylsulfonyl fluoride (final concentration 4 mM) and heating for 15 min at 80°C in a buffer containing 65 mM Tris-HCl, pH 7.8, 5% (v/v) 2-mercaptoethanol, 2% (w/v) SDS, 15% (v/v) glycerol and 0.5% (w/v) bromophenol blue.

After SDS gel electrophoresis [7], the fragments were electroblotted onto activated glass fiber sheets and investigated in a gas-phase sequencer (type 470A, Applied Biosystems) as in [8,9]. Phenylthiohydantoin derivatives of amino acids were identified and quantitated by HPLC [10].

3. RESULTS AND DISCUSSION

The present method has been used to determine amino-terminal sequences of proteolytic fragments of phytochrome in order to localize proteolytic cleavage sites, exposed regions of the native peptide chain and epitopes for monoclonal antibodies within the entire amino acid sequence of phytochrome [5,9]. For these purposes, determination of short sequences of only 3–6 amino acids proved to be sufficient. When we investigated a 16 kDa fragment obtained by subtilisin digestion, it turned out that its amino-terminus (at residue 212 of the complete chain) was close to a heterogenous site in the cDNA sequences: the predicted amino acid residue at position 220 was valine for clones AP3 and AP4 but methionine for clone AP5 [4]. We therefore determined the amino acid sequence of the first 10 residues of the 16 kDa fragment, i.e. up to residue 221 of the complete phytochrome sequence. The sequence is:

S L P G G S M E V/M L

It corresponds exactly with the predicted sequence derived from those cDNA clones which have already been sequenced, namely AP3, AP4 and AP5. There is no heterogeneity in steps 1–8 and 10. The finding of both valine and methionine at step 9 is the first proof of the expression of different phytochrome genes at the protein level. Since we have extensively purified our phytochrome sample, we must conclude that the different phytochrome isoproteins co-purify in our isolation procedure.

The analysis for heterogeneity is shown in more detail in fig.1. The expected values result from the repetitive yield (= 93.5% per Edman step). This value is reached by methionine in step 7 but is not

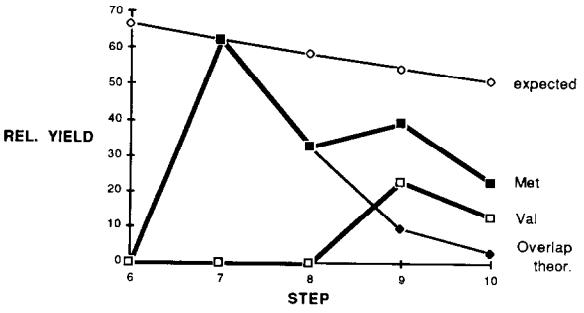

Fig.1. Heterogeneity of a 16 kDa subtilisin fragment from oat phytochrome in position 9. Only values for methionine (■) and valine (□) are given; values for glutamate (position 8) and leucine (position 10) are omitted.

reached by valine in step 9. The investigated peptide cannot contain only valine in position 9, therefore. Due to the overlap of phenylthiohydantoin derivatives, values do not decrease to zero within one step. The experimental value for the overlap of methionine is shown in step 8 and extrapolated further to steps 9 and 10. The difference between the theoretical overlap value and the experimental value for methionine in step 9 is the true value for methionine in this step. The approximate ratio valine/methionine is 1:1.3. This indicates that the isoprotein corresponding to clone 5 which only contains methionine in this position must be a major product of gene expression. Since more than 80% of the cDNA clones isolated were either of type 4 or 5 [4], one can conclude that the gene product corresponding to clone AP3 might be a minor product. Such a conclusion presumes, however, that the relative frequency of cDNA clones in the bacterial transformation reflects not only the relative proportion of the particular mRNA but also of the corresponding isoprotein.

The heterogeneity of the primary structure of oat phytochrome can eventually explain some of the heterogenous properties of phytochrome [3]. Its physiological significance remains unclear at present, however, especially since Lissemore et al. [11] did not find any evidence for the expression of multiple phytochrome genes in *Cucurbita*.

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