SOME FURTHER OBSERVATIONS ON PROTEIN FRACTIONS ISOLATED FROM WHEAT LEAVES SUBJECTED TO MOISTURE STRESS

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The soluble protein fraction from wheat leaves can be separated on agarose Bio-gel A-5m into two major fractions, one of very high molecular weight (probably exceeding 1 x 10^6) and the second of 150,000 to 250,000. The second fraction decreased markedly with drought stress, while the first fraction increased, especially after recovery from drought. A small molecular weight fraction which was further fractionated on Sephadex G-25 increased in droughted plants and consisted of free amino acids and probably nucleotides. It appears that the proteins in drought-stressed leaves are degraded to amino acids. No significant quantity of small peptides accumulated.

Some of the more striking metabolic changes in plant leaves subjected to moisture stress are those involving proteins (1). Stutt and Todd (2) demonstrated marked declines in total protein in the soluble fraction with apparent conversion to smaller proteins and amino acids. Gel electrophoresis indicated the appearance of faster moving bands, and enzyme activities were associated with certain bands (2,3). Fractionation with Sephadex G-100 gave a protein band emerging with the void volume, while a second fraction, which was greatly increased in stressed plants, was eluted in about the same volume as trypsin (mol. wt. about 20,000). Other workers (4-6) have reported accumulation of free amino acids in drought-stressed plants which we have confirmed in wheat (unpublished).

There was thus some question as to the nature of this low molecular weight, fluorescent, Folin-phenol positive fraction. The present study was initiated to clarify further the nature of this fraction and, using agarose gel, to obtain additional information concerning the high molecular weight fraction.

MATERIALS AND METHODS

Two hard red winter wheat varieties (*Triticum aestivum* L.) 'Ponca' and 'KanKing' were grown in 6 inch clay pots in vermiculite in a controlled temperature of 20 to 22 C with 14 hr daily illumination by Gro-lux fluorescent lamps giving about 11,000 lux at plant level. The plants were watered regularly with Hoagland's nutrient solution until the water stress period was started. Control plants received water daily and were 2 to 3 weeks old at time of sampling. Drought-treated plants did not receive water for about 15 days. Rewatered plants received the same treatment as droughted plants except samples for analysis were taken after plants had been allowed to recover from drought for 3 to 5 days. Relative water content was determined as described previously (7).

Sufficient leaf material was used to give an approximate dry weight of 0.1 g (actually determined each time on a comparable leaf sample). The material was ground in 2 ml of 0.1 M phosphate buffer pH 7.6, in a glass hand homogenizer and the homogenizer was rinsed with an additional 1 ml of buffer. The homogenate was centrifuged at 27,000 x g for 30 min. The supernatant was removed and enough sucrose added to make a 3% sucrose solution. Of this supernant, 0.3 ml was placed on either a 1.3 x 85 cm Bio-gel A-5m column (flow rate 0.6 ml/min) or a 1.3 x 60 cm Sephadex G-25 column (flow rate 0.4 ml/min). The material was eluted with the same buffer used to prepare the homogenate. Eluant from the Bio-gel column passed through a continuously recording fluorometer (Turner Model 111 equipped with a T-5 UV lamp; excitation filter 7-54; emission filters of 7-37 and 50%) followed by a fraction collector. For the

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Sephadex column, the fluorometer was read manually (254 nm interference excitation filter and emission filters 7-60 and 50%). Eluant was collected in 5 ml fractions. UV absorbance was measured at 280 nm with a spectrophotometer (either Beckman Model DU or Perkin-Elmer Model 202).

Supplies of the proteins used as standards were as follows: λ -globulin, cytochrome c, and catalase from Nutritional Biochemicals Co., Cleveland, Ohio; trypsin, pepsin, and tryptophane from Calbiochem, Los Angeles, Calif.; bovine serum albumin from Sigma Chemicals, St. Louis, Mo.; urease from Dr. G. Gorin, Department of Chemistry, and tobacco mosaic virus from Dr. D. Wadsworth, Department of Botany and Plant Pathology, Oklahoma State University.

RESULTS AND DISCUSSION

Soluble leaf proteins separated previously with Sephadex G-100 (2) gave a single peak eluting in the void volume of the column, indicating a molecular weight exceeding 100,000. In hopes of separating these high molecular weight materials, Bio-gel A-5m was chosen since it is purported to separate molecules in the range of 100,000 to 5,000,000. To check the resolving power of the column, a number of proteins of known molecular weight were compared. Tobacco mosaic virus (MW about 40 x 10^6) was assumed to move in the void volume (Vo). The elution volume (Ve) of the various proteins was determined and the ratio Ve/Vo was plotted against log molecular weight as shown in Fig. 1. This curve compares favorably with those of Determann (8).

When soluble proteins from wheat leaves were separated on Bio-gel A-5m, the first peak consistently eluted in the void volume indicating a high molecular weight (probably exceeding one million). A second peak emerged consistently at an elution volume of about 80-85 ml. If this is a spherical molecule without unusual binding properties, a molecular weight of 150,000 to 250,000 would be indicated. A third peak with greater UV absorption emerged at 125 to 130 ml. No conclusions can be drawn concerning the molecular weight of this fraction since it falls outside the resolution range of the gel.

Elution patterns of soluble proteins were similar for the two wheat varieties, and only those for variety 'Ponca' are shown in Figure 2. The second fluorescent peak decreased

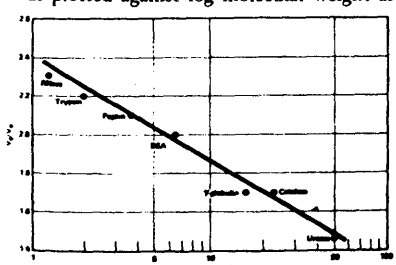

FIGURE 1. Separation of proteins according to molecular weight on Bio-gel A-5m agarose gel. V_e = elution volume of the protein shown; V_o = elution volume for tobacco mosaic virus.

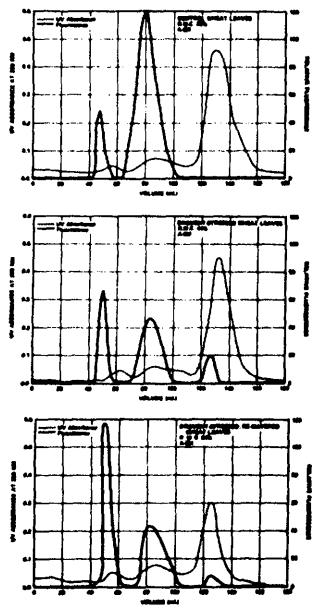

FIGURE 2. UV absorbance and fluorescence of fractions obtained after separation of soluble leaf proteins with Bio-gel A-5m agarose gel. R. W. C. = relative water content.

markedly with increasing moisture stress. This fraction did not return to the initial level after the droughted plants were rewatered. The Sephadex G-100 used earlier (2) would not have been expected to resolve the first and second peaks since the second fraction has an apparent molecular weight in excess of 100,000.

The third fluorescent fraction was not present in control leaves, but appeared after the drought stress was imposed. This fraction decreased somewhat when the leaves recovered following drought stress (Fig. 2).

To investigate further the nature of the smaller molecules present in this soluble fraction, a column of Sephadex G-25 was used. The first two fractions obtained on Bio-gel A-5m should be eluted in the void volume. The elution patterns obtained on Sephadex G-25 are shown in Figure 3 for leaves from stressed, non-stressed, and stressed-rewatered 'Ponca' plants. Most proteins were eluted in less than 50 ml as no visible precipitate formed with the addition of trichloroacetic acid with fractions eluted with more than 50 ml. This first fraction had both high UV absorption and flourescence. UV-absorbing fractions were obtained at 85-90 ml and 155 ml. Fractions giving fluorescence were found at 130 ml and 190 ml. Ninhydrin tests were made on the various fractions and maximum color was given by the fraction eluting in the void volume (the proteins) and 85 ml (corresponding to a UV absorbing peak). Lesser ninhydrin-positive reactions were given at 130 ml (fluorescence peak) and 155 ml (UV absorbing peak). The fluorescent fraction eluted with about 130 ml increased markedly upon drought and then declined again upon rewatering the droughted plants. The UV-absorbing fraction at 155 ml was present only in droughted plants and persisted after the plants had recovered.

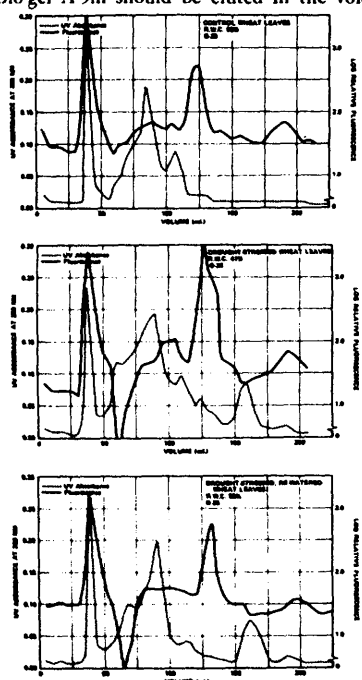

FIGURE 3. UV absorbance and fluorescence of fractions obtained after separation of soluble leaf proteins with Sephadex G-25. R.W.C. = relative water content.

Filters used for fluorescence determination on the fractions obtained on the Sephadex G-25 column were different than those used on the Bio-gel A-5m column, and it was noted that samples from droughted leaves separated on Sephadex G-25 yielded a fraction, eluting at 65 ml, that gave negative values. This result indicated the presence of a strongly UV-absorbing substance that does not fluoresce. From the spectrum and behavior on Sephadex, it seemed possible that this fraction might consist of free nucleotides. To check this possibility, ATP was placed on the column; it was eluted in 65 ml and gave a negative reading for fluorescence. Tryptophane, on this particular column, was eluted in 185 ml.

These results confirm those of others who have observed increases in free amino acids in leaves under drought stress (4-6). We have found similar increases in free amino acid content of drought stressed wheat leaves, especially proline and tryptophane (unpublished data of J. T. Cothren and G. W. Todd). The results also strongly indicate that the fraction from Sephadex G-100

designated "B" is not a small protein or peptide as implied earlier (2) but is comprised, in fact, of free amino acids and other small molecules. Gel electrophoresis has demonstrated some increase in smaller protein molecules, but evidently hydrolysis proceeds rapidly and, hence, these do not accumulate as free amino acids do.

ACKNOWLEDGMENT

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