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Evaluation of Foliar Application and Stem Injection as Techniques for Intrinsically Labeling Wheat with Copper-65

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Foliar application and stem injection were evaluated as techniques for intrinsically labeling wheat with ^{65}Cu. The copper concentrations in the whole wheat flours were as follows: 9.1 $\mu g/g$, control; 12.9 $\mu g/g$, foliar application; 22.1 $\mu g/g$, stem injection. Four protein fractions (globulins and albumins, glutenins, gliadins, remaining proteins) were sequentially extracted from defatted whole wheat flours. Gel column chromatography of the fractions demonstrated that the elevated concentrations of copper in the foliar-applied and stem-injected wheat did not alter any of the significant proteins based on V_e/V_0 values. These results indicate that the bioavailability of copper should not differ among the control and the ^{65}Cu-treated wheats.

INTRODUCTION

Copper is widely distributed in foodstuffs, but information on the proportion of dietary copper actually absorbed is limited. A daily copper intake of 2–3 mg is recommended for adults (National Academy of Sciences, 1980), but dietary surveys show that actual intake is often below 1 mg/day (Klevay, 1975). Absorption of copper as $CuCl_2$ from purified diets (Turnlund et al., 1982; Turnland, 1984; King et al., 1978) and in the fasting state (Johnson, 1984) has been studied in humans, but the bioavailability of copper to humans from foodstuffs is virtually unknown. With the increased emphasis on determining the bioavailability of trace elements from major foodstuffs, intrinsic labeling of foods with isotopes has become essential.

Previous work in this laboratory demonstrated that sufficient enrichment of wheat with ^{65}Cu to be used in human bioavailability studies can be obtained by stem injection or foliar application of ^{65}Cu (Lykken, 1984). But the question of whether these techniques altered the distribution of Cu or the protein composition of wheat remained unanswered. This study was initiated to evaluate foliar application and stem injection of ^{65}Cu as techniques for intrinsically labeling wheat using Cu distribution and the composition of various protein fractions as the major criterion for comparison.

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METHODS AND MATERIALS

Wheat, *Triticum aestivum* var. Waldron, was grown in a greenhouse with supplemental lighting provided by 400-W high-pressure sodium lamps (Energy Technics, York, PA) to produce a 16-h light/8-h dark cycle. Plants were grown in 8-in. plastic pots, seven plants per pot, in soil with 30% perlite. A total of 70 plants were grown for each of the control and foliar-application and stem-injection treatments. Plants were watered and fertilized (20:20:20) (Peter's Fertilizer, W. R. Grace and Co., Fogelsville, PA) as needed.

Since copper has only two short-lived radioisotopes, ^{64}Cu ($t_{1/2}$ = 12.0 h) and ^{67}Cu ($t_{1/2}$ = 61.9 h), only the stable isotope, ^{65}Cu, is suitable for studies of copper absorption in humans. Thus, ^{65}Cu as $^{65}CuCl_2$ (Oak Ridge National Laboratory, Oak Ridge, TN) was applied to wheat plants as outlined below. Foliar application of ^{65}Cu during anthesis was performed as follows: $^{65}CuCl_2$ was added to a citrate–phosphate buffer (McIlvaine, 1921), pH 5.5, plus 1% sodium lauryl sulfate. The final concentration of ^{65}Cu was 0.3 mg/0.7 mL of solution. This solution (0.7 mL/plant) was sprayed onto the plants with a hand-held plant sprayer. A published method was used for stem injections of $^{65}CuCl_2$ at a rate of 0.3 mg of ^{65}Cu/plant (Starks and Johnson, 1985). In this study 0.2 mL of the solution was injected compared to the 0.7 mL previously reported

Table I. Copper Content of Whole Wheat Flour and Protein Extracts (μg/g)

			extr from defatted flour			
treatment	whole wheat flour[a]	fat extr	globulins and albumins	gliadins	glutenins	extract D
control	8.1 ± 0.4	0.4 ± 0.0	1.50 ± 0.21	*b*	2.00 ± 0.0	2.50 ± 0.21
foliar	12.9 ± 0.3	0.9 ± 0.1	2.00 ± 0.32	*b*	2.50 ± 0.21	5.00 ± 0.0
stem inj	22.1 ± 0.6	0.8 ± 0.2	2.67 ± 0.0	0.77	8.50 ± 2.31	4.00 ± 0.0

[a] Before defatting. [b] Below detectable limits.

(Starks and Johnson, 1985).

After harvesting, the seeds were separated from the chaff by hand and ground in a coffee and spice mill. The total copper content was determined for each treatment (control, foliar application, stem injection) in triplicate by using standard atomic absorption spectrophotometric methods after ashing with nitric acid and hydrogen peroxide.

The remaining whole wheat flours were defatted with butanol and petroleum ether. Two portions of the defatted flour from each treatment were sequentially extracted for proteins (globulins and albumins, gliadins, glutenins, remaining proteins) and lyophilized (Starks and Johnson, 1985).

A 30-mg sample of each of the lyophilized extracts was redissolved in 1 mL of the appropriate buffer and subjected to gel filtration chromatography. The 0.04 M NaCl extracts (globulin and albumins) were chromatographed on a Sephadex G-75 column, 1.5 × 90 cm, with a phosphate buffer, pH 7.0. The 70% ethanol extracts (gliadins) were chromatographed on a G-25 column, 1.5 × 90 cm with a phosphate buffer, pH 7.0. The 0.1 N acetic acid (glutenins) and the D extracts (remaining proteins) were chromatographed on a Sephadex G-50 column, 2.0 × 40 cm, with an acetate buffer, pH 4.65. Each extract was eluted twice on the proper column. The eluted fractions were passed through a UV single-path monitor at 280 nm to determine protein content, and thus the values reported are means of four runs for each extract. All flow rates were 1 mL/4 min. After the void volume, 160 mL of eluted substances was collected for each run. The 1-mL fractions were analyzed by atomic absorption spectrophotometry to determine the copper content.

Samples of each protein extract were wet ashed with nitric acid and hydrogen peroxide, and copper was determined by atomic absorption spectrophotometry.

RESULTS AND DISCUSSION

Efficiently labeling foodstuffs with stable isotopes is a prerequisite for conducting bioavailability studies in humans. Comparison of the total copper in the three treatments reflected the relative efficiency of the techniques (Table I). Weaver (1984) has stated that foods must be highly enriched with a stable isotope to be useful in bioavailability studies. This is particularly true with copper since the natural ratio of ^{63}Cu to ^{65}Cu is 69.09 to 30.91. Human copper absorption studies require the feeding of 1–3 mg of ^{65}Cu in excess of its natural abundance in food (Johnson, 1984; Turnlund, 1984). Maximal enrichment of plant tissues would be achieved in a hydroponic nutrient solution in which the stable isotope replaced the natural mineral (Weaver, 1984), but considerable amounts of copper accumulate and are retained in plant roots (Tukendorf et al., 1984). Furthermore, the copper retained in the roots is absorbed or strongly bound with proteins and deposited in cell membranes and intracellular spaces (Woolhouse and Walker, 1981). For these reasons, addition of ^{65}Cu to a hydroponic solution would not be the most efficient use of stable copper and could be prohibitively expensive in the quantities needed for human

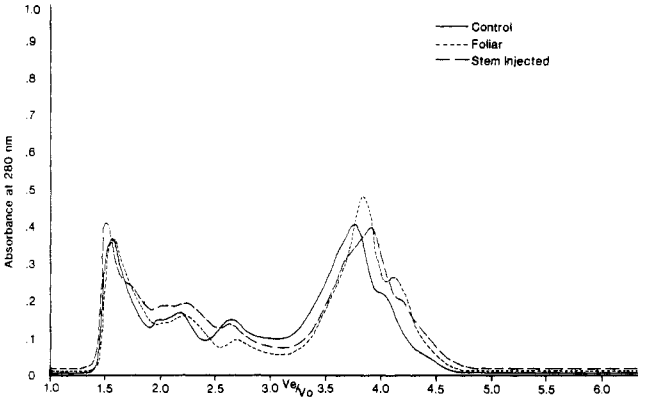

Figure 1. Chromatograms of globulin and albumin extracts.

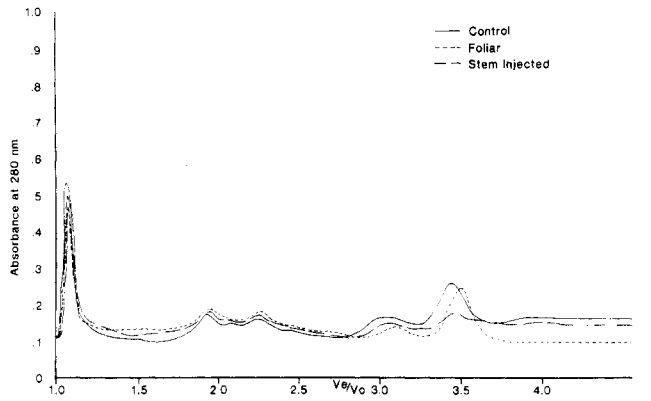

Figure 2. Chromatograms of gliadin extracts.

bioavailability studies. Thus, alternative methods such as foliar application and stem injection of isotopes into plants needed to be developed.

The average gross composition of wheat grains (percent dry weight basis) is as follows: protein, 12.2; fat, 1.9; starch, 71.9; fiber, 1.9; ash, 1.7 (Lasztity, 1984). It has been shown that two copper proteins, platocyanin and superoxide dismutase, account for 60 to >90% of the total copper content of barley and other species leaf cells (Hewitt, 1983). In mammalian systems, several proteins such as ceruloplasmin, cytochrome oxidase, monoamine oxidase, tyrosinase, superoxide dismutase, etc., require copper as part of their molecular structure (Evans 1973). Thus, it seems likely that copper in wheat grain might be associated with a particular protein or proteins.

Because only low levels of copper were found in the fat portion of the grain and none was detected in the carbohydrate residue after protein extraction (Table I), the protein fraction of the grain was sequentially extracted to determine the effect of the various copper treatments on the different classes of proteins. The protein fraction accounted for 75.0, 73.6, and 72.1% of the total copper content of the whole wheat flour for the foliar-treated, stem-injected, and control wheat, respectively.

The higher concentration of copper in foliar-treated and stem-injected plants was reflected in the various protein

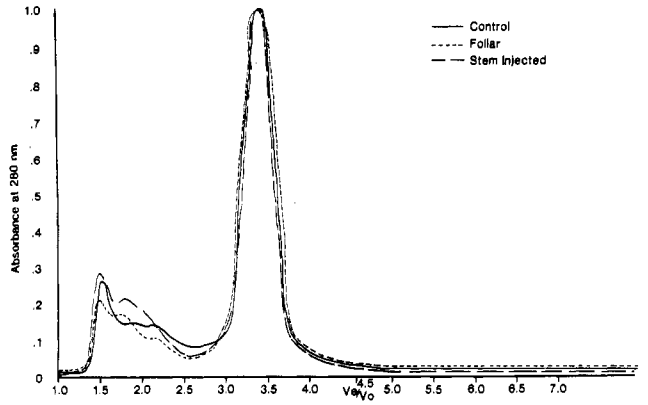

Figure 3. Chromatograms of glutenin extracts.

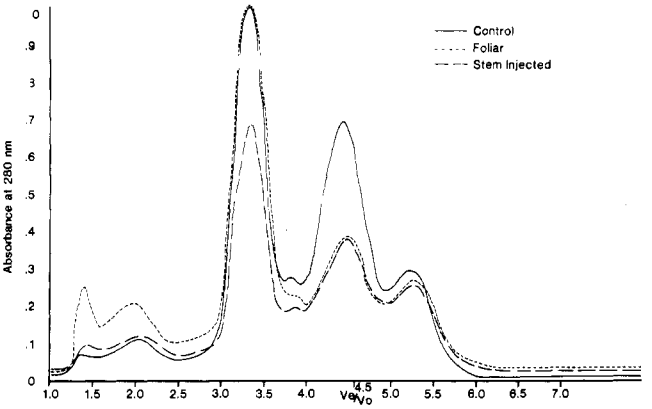

Figure 4. Chromatograms of remaining proteins (extract D).

classes (Table I). Wheat gluten, which is related to flour's rheological and baking properties, is composed of gliadin and glutenin (Danno et al., 1974). As was the case with ^{65}Zn (Starks and Johnson, 1985), the greatest amount of copper was in the glutenin fraction, although this fraction represents only 30–40% of the total protein (Larkins, 1981). The gliadin fraction, which constitutes 40–50% of the total protein (Larkin, 1981), contained undetectable or minimal levels of copper (Table I).

To determine whether the higher levels of copper produced by foliar application or stem injection had altered the protein content or distribution, the four protein fractions were chromatographed on Sephadex (Figures 1–4). The eluted 1-mL fractions were analyzed for copper content. All 1-mL fractions had copper levels that were below detectable limits.

The chromatograms were compared by using the V_e/V_o ratios where V_o, the void volume, is the elution volume for a substance that is completely excluded from the gel and V_e is the measured elution volume of the sample.

The chromatograms of the globulin and albumin fractions were very similar among treatments (Figure 1). The V_e/V_o values for the various protein peaks were identical, except for a slight shift in the peaks with V_e/V_o values of 3.71–3.87 (Figure 1). These peaks consisted of substances with molecular weights of <100 and were probably amino acids. In addition, the foliar-treated wheat had a small peak at $V_e/V_o = 4.17$. This peak may have been due to adsorption of these small molecular weight proteins or amino acids to the Sephadex gel.

The gliadin fractions of the three treatments had very similar chromatograms (Figure 2). As with the globulin and albumin fraction, the only difference was in the very small molecular weight substances ($V_e/V_o = 3.42$, $M_r <$ 100).

Chromatograms of the glutenin fraction showed some differences in the intensity of the first protein peak ($V_e/V_o = 1.52$, $M_r \approx 7200$), but the differences were slight. A slight peak in the foliar-treated and stem-injection fractions at $V_e/V_o = 1.75$, $M_r \approx 3000$, was a variation from the control (Figure 3). Tukendorf et al. (1984) reported stimulation of protein synthesis in spinach by excess copper in a hydroponic nutrient solution. Such a phenomenon may have caused this additional protein or produced more of this protein so that it was detectable.

In a related study, Ragasits and Varga (1978) published elution diagrams of glutenin extracts on Sephadex G-100 columns. The results showed significant changes in both high and low molecular weight protein fractions of wheat grown under different levels of fertilization with N, P, and K fertilizers. These changes were much greater than the slight changes observed in the glutenin fraction of the various copper treatments in this study.

The remaining protein fractions (extract D) from the three treatments had similar chromatograms. All the protein peaks had identical V_e/V_o values, with the only differences being variations in the intensity of the peaks (Figure 4). These differences may be explained by the findings of Tukendorf et al. (1984) because the first peak ($V_e/V_o = 1.46$, $M_r \approx 9100$ was greater in the foliar wheat (Figure 4) and the copper content in this fraction was greater in the foliar-treated wheat when compared to the control and stem-injected treatments (Table I). The differences in the peaks at $V_e/V_o = 3.32$ and 4.50 presumably were not due to excess copper stimulation. The importance of these small molecular weight peaks ($V_e/V_o = 3.32$, $M_r < 100$; $V_e/V_o = 4.50$, $M_r < 100$) is probably limited. These were most likely amino acids or other small molecules since oxytocin ($M_r \approx 1000$) had a V_e/V_o value of 2.35. But although the peak intensities varied, the V_e/V_o values remained identical for all treatments.

The results of this experiment demonstrated that foliar application and stem injection are reliable means of intrinsically labeling wheat with stable isotopes. The elevated concentrations of copper in the foliar-applied and stem-injected wheat did not significantly alter the protein distribution and composition as assessed by gel chromatography. One might assume that the bioavailability of copper would not differ among the treatments since wheat grain ranges from 9.0 to 18.0% protein (Lasztity, 1984) and the majority of the copper (72.1–75.0%) was found in the protein fractions. Even the nearly threefold increase in total Cu in the stem-injected wheat over the control did not significantly change the protein composition of the glutenin fraction and only a small change in the amount of the high molecular weight component of the gliadin fraction. Stem-injected wheat with a total Cu content of 12 ppm and an enrichment of 74 atom % ^{65}Cu has been grown and used in a study of Cu absorption in humans (Johnson, 1985). Presumably, the protein composition of that wheat was even more similar to control wheat than was the stem-injected wheat studied here. Finally, stem injection and foliar application are less costly than hydroponically applying expensive stable isotopes, and they ensure that the majority of the applied isotope is deposited in the edible portion of the plant.

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Removal of Nucleic Acids from Yeast Nucleoprotein Complexes by Sulfitolysis

Srinivasan Damodaran

A simple method for removal of nucleic acids from yeast nucleoprotein complexes by sulfitolysis is described. Treatment of yeast nucleoproteins with sodium sulfite followed by sodium tetrathionate caused destabilization and dissociation of nucleoprotein complexes. Subsequent precipitation of proteins at pH 4.2 resulted in a protein preparation with low levels of nucleic acids. A good correlation between the extent of nucleic acid removal and the disulfide content of the sample was also observed. However, when the nucleoproteins were treated with either sodium sulfite or sodium tetrathionate only, there was no appreciable removal of nucleic acids. Further, the efficiency of nucleic acid removal by sulfitolysis also depended on the initial nucleoprotein concentration. The mechanism of dissociation of nucleoproteins by sulfitolysis and the advantages of sulfitolysis over those of other chemical modification procedures to reduce nucleic acid content in single cell proteins are also discussed.

INTRODUCTION

One of the major problems limiting the exploitation of proteins from microbial sources is the high level of nucleic acid contamination in these protein preparations (Sinskey and Tannenbaum, 1975; Vananuvat and Kinsella, 1975; Lipinskey and Litchfield, 1974). From nutritional point of view, consumption of high levels of nucleic acids in the diet (above 2 g/day) causes disorders such as urecemia, gout, and kidney stone formation (Miller, 1968; Waslein et al., 1970). Hence, in order to develop single-cell proteins for human nutrition, it is imperative to develop methodologies to reduce the nucleic acid content of the isolated protein to a safe level.

There are several methods currently available for the removal of nucleic acids from single-cell proteins (Newell et al., 1975a,b; Robbins et al., 1975; Shetty and Kinsella, 1979). However, most of these methods have major disadvantages in terms of functional quality and nutritional safety of the isolated proteins. For example, alkaline hydrolysis of nucleic acids at elevated temperatures (Newell et al., 1975b) not only results in thermal denaturation and impairment of functional properties of the protein but also causes destruction of essential amino acids and formation of potentially toxic compound known as lysinoalanine (Shetty and Kinsella, 1980). Similarly, enzymatic hydrolysis of nucleic acids results in concomitant degradation of proteins by endogenous proteases (Lindbloom, 1977). Chemical modification of yeast nucleoproteins with acyl anhydrides has been shown to decrease the nucleic acid level (Shetty and Kinsella, 1979). However, such modifications impair bioavailability of lysine. Furthermore, the biological safety of isopeptides, e.g., succinyllysine is not known. Recently, a simple and safe method using chaotropic salt treatment to reduce the nucleic acid contents in yeast protein isolates has been described (Damodaran and Kinsella, 1983a,b). However, the practicality of this approach needs further studies.

In our continuing effort to develop simple methods for the isolation of yeast proteins with low levels of nucleic acids, we studied the effect of sulfitolysis on the dissociation of yeast nucleoprotein complexes. The basic principle involved in this approach is that conversion of sulfhydryl and disulfide groups in nucleoproteins to *S*-sulfonate derivative would cause conformational changes and also increase the electronegativity of the protein

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