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Rapid Estimation of Potato Tuber Total Protein Content with Coomassie Brilliant Blue G-250*

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Summary. A new method for measuring protein with Coomassie Brilliant Blue G-250 has been adapted for use as a screening method in a potato tuber protein improvement breeding program. The method is simple, fast and inexpensive, and has successfully estimated the total protein content of a broad range of tuber genotypes having dissimilar amino acid profiles and tuber maturities. Correlation between the Coomassie method and a modified micro-Kjeldahl method, the standard method used in the potato breeding program, was 0.93. Free amino acids and other compounds which interfere with other methods for measuring protein do not interfere with the Coomassie Brilliant Blue procedure.

Key words: Protein Breeding – Protein Extraction – *Solanum tuberosum* L. – Protein Selection Method – Protein Dye-Binding

Introduction

Efficient genetic selection for increased protein content in potatoes has been hampered by the lack of an inexpensive, rapid, and reliable method for determining protein. Bradford (1976) has recently published a method for measuring protein based on a color shift that occurs when Coomassie Brilliant Blue G-250 (CB) binds to protein. Five micrograms of protein per ml of test solution gave an absorbance of .275 units, which was five times as sensitive as the Lowry procedure. Slightly different absorbance values resulted when equal quantities of different proteins reacted with CB. Bradford (1976) suggested that this variation resulted from difficulties in determining the exact amount of protein in a solution (inaccurate extinction coefficients) and differing efficiencies among the various proteins to bind CB. Even though minor variations occurred, Bradford's data demonstrated that more variation occurred when the same proteins were quantified with the Lowry procedure.

Sensitivity and only minor variation of binding to different proteins are not the only virtues of the CB procedure. This remarkably simple procedure involves few steps: five ml of the CB reagent are added to a small aliquot of solution containing protein and then the absorbance of the resulting solution is read within 60 minutes at 595 nm.

Because the CB procedure has sensitivity, slight variation with different proteins, and simplicity, we wanted to evaluate the procedure as a possible alternative to the modified micro-Kjeldahl procedure currently used for determining protein content in our segregating populations. The CB procedure has to meet several criteria before we can accept it as a viable alternative:

A. Tuber protein must be completely solubilized and in a form compatible with the CB reagent.
B. Protein extraction must be efficient, regardless of the stage of tuber development or genotype.
C. The CB reagent must react similarly with all tuber protein regardless of genotype or stage of development.
D. The CB procedure must reliably predict protein content over a broad range of protein concentrations.
E. The CB reagent must not react with free amino acids or extraneous substances in the tuber.
F. The CB procedure must be faster and less expensive than our current ethanol-wash, micro-Kjeldahl procedure.

We decided that we could judge whether or not some criteria were met on the basis of the agreement between a standard curve and a curve generated from the regression of CB absorbance values on protein content obtained with the modified micro-Kjeldahl procedure. We also decided

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that the ability of CB to bind to free amino acids would be directly assayed.

Materials and Methods

Potato tubers were cut, frozen, lyophilized, and ground as previously described (Desborough 1975). Protein was determined by micro-Kjeldahl and is the N content of the 80% ethanol insoluble portion of the tuber dry matter, multiplied by 6.25 and expressed as a percentage of whole tuber powder.

L-amino acids and Coomassie Brilliant Blue G-250 (CB) were obtained from Sigma Chemical Co. Dye content of the CB was approximately 65%.

Procedures for making the CB reagent are described by Bradford (1976). Procedures for determining protein with CB are similar to those of Bradford (1976) except for the volume of the aliquot taken for analysis (0.1 versus 0.4 ml).

For the standard curves bovine serum albumin (BSA) (Miles Laboratories Inc.) was dissolved in water. The concentration of BSA in this solution was determined by extinction coefficient methods (ϵ_{280} = 6.6) (Kirchenbaum 1970). This solution was then diluted with 1 N NaOH to give a final concentration of 0.5 N NaOH and subsequently used to construct the standard curves.

Breeding methods, parentage of the hybrids, and examples of the variability of tuber protein content and the amino acid content of the protein have been published elsewhere (Desborough and Lauer 1977; Desborough and Weiser 1972; Desborough and Weiser 1974).

Results

Ability of CB to Bind Free Amino Acids. For this portion of our study, twenty L-amino acids were used: Ala, Val, Leu, Pro, Phe, Trp, Met, Gly, Ser, Thr, Cys, Tyr, Asn, Gln, Asp, Glu, Lys, Arg, and His. Two mg of each amino acid were dissolved in five ml of water. A 0.4 ml aliquot was removed from each of these solutions, placed in a test tube, and then five ml of the CB reagent were added. Absorbance of the resulting solution was read at 595 nm on a spectrophotometer. The solution containing the aliquot of isoleucine had the greatest absorbance – 0.003 units. The concentration of each amino acid in the final solution exceeded by a factor of 4 to 100 the concentration of the amino acid that occurs when 15 mg of tuber powder is assayed by the normal procedure (5 ml NaOH, 0.4 ml aliquot). Even at these excessive concentrations, free amino acids do not interfere with the estimation of protein with CB.

Standard Curve Generated from Bovine Serum Albumin (BSA). Fig. 1 illustrates the standard curve derived from the reaction of BSA with CB. Bradford (1976) noted that the standard curve departs significantly from linearity. However, when the $\log_{10}$ of absorbance is plotted against the $\log_{10}$ of protein, the standard curve approaches linearity over the range of 0 to 200 µg/5.4 ml. Using this transformation, the data can be subjected to a linear regression analysis which results in a mathematical equation that will directly estimate the amount of protein from the absorbance value (Fig. 1) and eliminates the necessity of reading protein values directly from a standard curve as suggested by Bradford (1976).

Extraction and Solubilization of Potato Protein. We initially utilized NaOH as an extractant. We added 5 mls of 0.5 N NaOH to 15 mg of tuber powder in a test tube while agitating the tube on a Vortex Genie® mixer. Agitation avoided the formation of a gel which would adhere to the walls of the tube. A 0.4 ml aliquot of the extract was immediately removed, placed in another test tube, and 5

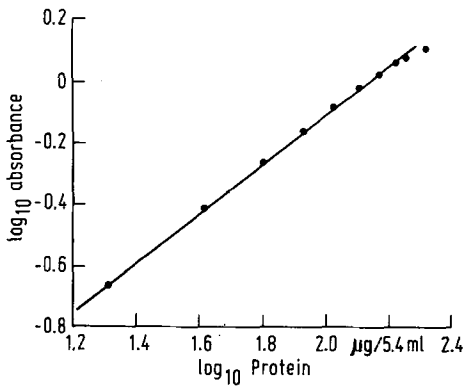

Fig. 1. Standard curve generated with BSA and plotted on a log-log basis. Regression equation for the data is $\log_{10}$ (absorbance) = $-1.73 + 0.815 \log_{10}$ (µg protein/0.4 ml aliquot). Each data point represents the average of four separate determinations

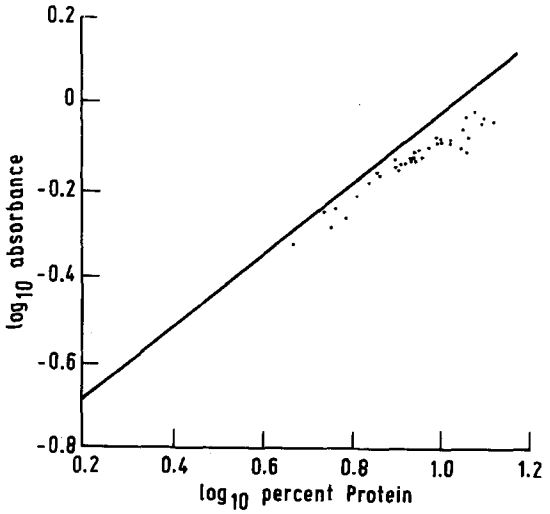

Fig. 2. The log of the absorbance of 40 samples plotted against the log of protein content (see text). The line represents the standard curve based on BSA. Correlation between protein content and absorbance = 0.92. Each data point represents the average of at least two separate determinations

ml of CB reagent was added. The absorbance of the resulting solution was read at 595 nm within 60 minutes. Absorbance values correlated highly with the percentage of protein determined by micro-Kjeldahl but the line formed by regression of absorbance on percent protein did not coincide with the standard curve, expecially at the higher protein percentages (Fig. 2). The lack of coincidence suggests that either extraction was not complete when we removed the 0.4 ml aliquot for analysis, or potato protein and BSA react dissimilarly with CB.

We used several approaches to determine whether or not incomplete extraction of the protein caused the lack of coincidence between the standard curve and the regression of absorbance on protein. Heating the tubes containing the NaOH and tuber powder for 5 minutes at 60°C or boiling them for 1 minute did not increase the resulting absorbance readings (data not included). However, the amount of time between adding the NaOH and removing the 0.4 ml aliquot significantly increased the absorbance readings (Table 1). Most of the increase occurred within 30 minutes after adding the NaOH and the absorbance readings virtually plateaued after 2 hours. We concluded that NaOH does not interfere with the binding of CB to protein and that the tuber powder must remain in the NaOH for at least 2 hours to obtain maximal readings.

We then added 5 ml of 0.5 N NaOH to 15.0 mg of tuber powder obtained from each of the 228 diverse genotypes described in Table 2. After adding the NaOH every sample was allowed to sit at room temperature for 2.5 hours before removing the 0.4 aliquot for assay with CB. Absorbance values correlated highly (r = 0.93) with percent protein (Fig. 3). Standard error of a mean was ± .011 absorbance units (about 0.1% protein). Micro-Kjeldahl values were not duplicated, but the standard error of a mean is usually ± 0.65 to 0.85% protein. Thus, 0.93 was as high a correlation as possible. Data points included in Fig. 3 are scattered around the standard curve and no trends in the scattering were associated with any of the groups listed in Table 2. Most likely the scattering was associated with the imprecision of the micro-Kjeldahl method. We concluded that the CB procedure reliably predicts tuber protein content among diverse genotypes after a 2.5 hour extraction period.

Table 1. Absorbance of solutions resulting from the addition of a 0.4 ml aliquot of tuber powder extract to 5 ml of CB reagent. Aliquots were removed at the indicated times after adding 0.5 N NaOH. Each datum is the average of two determinations. Each sample was a different *Tuberosum-Andigena* hybrid. Among the six hybrids, protein content, as measured by the modified micro-Kjeldahl, ranged from 5.8 to 12.3 percent

Sample Number	Minutes after adding NaOH				
	0	15	75	105	135
1	.697	.769	.767	.804	.797
2	.592	.606	.641	.648	.649
3	.604	.599	.613	.619	.633
4	.680	.715	.729	.750	.747
5	.546	.562	.594	.585	.584
6	.937	.984	1.019	1.052	1.046

Table 2. Genetic constitution, ploidy, and protein content, as measured by the modified micro-Kjeldahl method, of the individuals used for the comparison between the CB procedure and the modified micro-Kjeldahl

Genetic background (Groups within *S. tuberosum*)	Ploidy	Number of Samples	Range of Protein Content
Andigena X (Tuberosum X Phureja)	4x	26	4.9 – 10.6
Tuberosum	4x	21	5.5 – 11.9
Andigena	4x	93	3.0 – 13.2
Tuberosum X Phureja	2x	31	9.5 – 15.6
Tuberosum X Phureja	4x	57	5.5 – 10.9

Discussion

Breeding for increased protein in many field crops has been facilitated by the availability of efficient screening methods. Most of these methods have not been applicable to potato because tubers, as protein sinks, differ radically from the seeds of grains and legumes. Grain and legume seeds generally contain small numbers of storage or reserve proteins (Altschul et al. 1966) having little or no

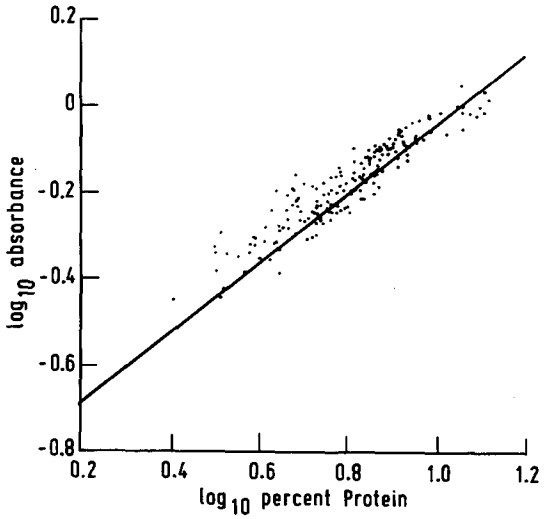

Fig. 3. The log of absorbance from 228 samples (Table 3) plotted against the log of protein content (see text). The line represents the standard curve based on BSA. Correlation between protein content and absorbance = 0.93. Each data point represents the average of at least two separate determinations

enzymic activity (Nelson 1970). Genetic variation for the amino acid composition of the storage protein is low (Johnson and Lay 1974; Swaminathan et al. 1969; Woodham 1973). Seeds, normally harvested at a definite stage of maturity, characteristically contain inconsequential quantities of free amino acids (Campang et al. 1971; Mica et al. 1975; Perez et al. 1973). In contrast, the proteins contained in a tuber are extremely diverse (Stegemann and Loescheke 1976) and none have been identified as having solely a storage protein function (Stegemann 1975). In tubers, free amino acids constitute from 40 to 70% of the reduced nitrogen (Li and Sayre 1975). Environment or stage of the development can influence tuber protein content (Desborough and Lauer 1977; Snyder et al. 1977), protein quality (Snyder et al. 1977; Stegemann 1975) and the size (Hoff et al. 1971; Li and Sayre 1975; Snyder et al. 1977) and composition (Coutrez-Geerinek 1975; Hoff et al. 1971) of the free amino acid pool. In populations segregating for protein content, variations in protein quality (Desborough and Lauer 1977) and the stage of tuber development at harvest also occur. Clearly, the methods used to measure protein in these diverse plant materials must fulfill different criteria. Breeding for increased tuber protein content requires an estimate of protein content that is reliable in spite of different amino acid profiles of the protein, the quantity and composition of the free amino acid pool, and the extractability of the protein among genotypes and developmental stages.

Because the amino acid content of the tuber protein varies among genotypes (Desborough and Lauer 1977), any test, such as the Orange G procedure (Udy 1956), based on the reaction of a compound with one or several specific amino acid residues would not give reliable estimates of protein content unless these specific amino acids correlate highly with protein content in every genotype generated. A Kjeldahl (Desborough and Weiser 1972) analysis of whole tuber powder does not reliably estimate protein content because the size of the free amino acid pool varies among genotypes, environments, and developmental stages. Non-proteinaceous substances in a tuber react with reagents used in the Lowry and biurette procedures (Potty 1969) which render these methods unreliable for estimating protein content. Desborough (1975) has demonstrated that the procedure based on the non-specific binding of bromo-phenol blue to potato protein (Swaminathan et al. 1973) does not reliably estimate protein content of potato tubers from diverse genotypes. Methods based on soluble protein (van Gelder and Krechting 1973; Hoff 1975), generally defined as that protein contained in expressed tuber sap, do not give reliable estimates because soluble protein does not correlate with total protein (Desborough and Weiser 1972).

In the past we have used total amino acid analysis of dialyzed tuber powder samples (Desborough and Weiser 1974) as a standard for judging the reliability of other techniques. Total amino acid analysis is too expensive and slow for routine use in a protein breeding program. The Potty (1969) procedure correlates highly with total protein-bound amino acids. The Potty method, also relatively slow and not suitable for a breeding program, eliminates interference in the biurette procedure caused by non-proteinaceous tuber components. We currently estimate the total tuber protein content with a modified micro-Kjeldahl procedure (Li and Sayre 1975; Snyder et al. 1977). The procedure estimates the N content of tuber tissue after the free amino acids are removed with 80% ethanol. Data resulting from this procedure correlate highly with either amino acid content of dialyzed tuber powder or protein determined by the Potty procedure (Li and Sayre 1975; Vigue and Li 1975). The modified micro-Kjeldahl procedure is slow (15 to 20 samples per man-day), subject to error because of the number of steps involved in each determination, and expensive (in excess of $3.00 per sample after sample drying and grinding). Clearly, the modified micro-Kjeldahl does not meet our needs for low cost and rapidity. In comparison, each determination done with the CB procedure costs about $.30 and 90 to 120 determinations can be completed per man-day.

In conclusion, the CB procedure meets all of the criteria listed earlier in this paper and exceeds the modified micro-Kjeldahl procedure in precision, rapidity and economy. Because the CB procedure successfully estimated the protein content of tuber powder from diverse genotypes representing many developmental stages and protein of differing amino acid compositions, and in the presence of plant substances which interfere with other procedures, the CB procedure has been adopted in our breeding program and has potential applications for measuring protein content in other species of plants.

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