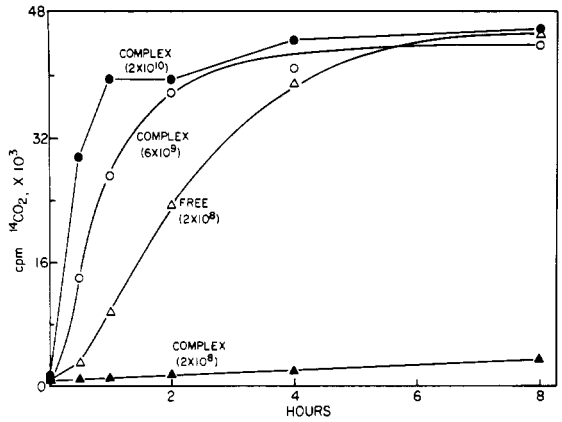

Figure 5. Evolution of $^{14}CO_2$ in the bacterial decomposition of bentonite–*n*-[1-^{14}C]decylamine complex and free *n*-[1-^{14}C]decylamine in inorganic salts solution with various initial bacterial cell densities (cells/mL).

microorganisms converted to cpm of $^{14}CO_2$, which was found to be 48% in 24 h in the clay-free solution. The calculations were based on the conversion for the clay-free solution because the total cpm in the bentonite–*n*-[1-^{14}C]decylamine solution could not be measured directly because of possible strong trapping of emitted beta particles within the clay lattice, where much of the amine was presumably located. For the clay suspensions, the calculated rates are: 10.5 μg mL^{-1} h^{-1} for 2×10^{10} cells/mL for the first 30-min period, 4.9 μg mL^{-1} h^{-1} for 6×10^9 cells/mL for the first 30 min, and 0.075 μg mL^{-1} h^{-1} for 2×10^8 cells/mL for the first 8 h. The clay-free solution with an initial bacterial density of 2×10^8 cells/mL had a mineralization rate of 2.4 μg mL^{-1} h^{-1} for the 30–120-min period.

Based on these calculations, it appears that the biodegradation rate at the two highest cell densities exceeds the highest rate of amine removal found here by factors of more than 5 and 10. These experiments were performed under the same conditions of temperature, amount of bentonite–decylamine/mL of suspension, and percent solids so that comparisons should be valid. The chief difference between the experiments involving bacteria and no bacteria was the degree of agitation of the suspension, which was much higher in the case of desorption with no inoculum. If the degree of agitation or shaking was increased for the suspensions containing the inocula, it is likely the rates of CO_2 production would have been even higher and the discrepancy between the rates of degradation and removal even larger.

These observations suggest that the desorption rate does not limit the rate of biodegradation of the bound *n*-decylamine, and the mineralization rate of this sorbed compound is not independent of inoculum size at high cell densities. The microorganisms thus may facilitate removal of the decylamine from the clay surface; e.g., by production of extracellular enzymes or by bringing about a pH change at the clay surface. Nevertheless, CO_2 production from the sorbed decylamine is markedly lower than from the free amine. The reason for this reduction in rate is not clear, but it is not the result of a toxicity of the clay because the rate of $^{14}CO_2$ production from D-[^{14}C(U)]glucose by two pure bacterial cultures isolated from the mixed culture growing on *n*-decylamine was the same in the presence and absence of bentonite (0.014% solids). D-[^{14}C(U)]Glucose did not adsorb to the clay. The bound decylamine may be degraded more slowly than the free amine because some portion or all of that which is bound on the inner clay surfaces is poorly accessible to the microorganisms.

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Processing of Fresh Tobacco Leaves for Protein Fractions

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A scaled-up process was developed for the preparation of crystalline fraction I protein and fraction II protein from tobacco. Laboratory tests were used to evaluate possible conditions for pilot plant processing. The plant tissue was treated with a reducing agent to inhibit undesirable oxidative reactions. Sephadex G-50 was used for large-scale gel filtration. The yield of crystalline fraction I protein was greater from leaf tissue than from stem tissue (3 g/kg of fresh leaf and 0.02 g/kg of fresh stem). Forty-five kilogram batches of fresh leaf tissue could be processed to yield 95 g of crystalline fraction I protein, which had a nitrogen content of 17.03%. The dried protein fractions were analyzed for amino acid content and soluble nitrogen.

In anticipation of increased needs for edible protein, research is being conducted on more efficient utilization of the protein that is produced by green plants. Much plant protein is converted to animal protein with considerable loss before it is consumed by humans as meat, milk, and eggs. Greater efficiency can be achieved by extracting protein concentrates from green leafy tissue and using the fibrous residue, which still contains some protein,

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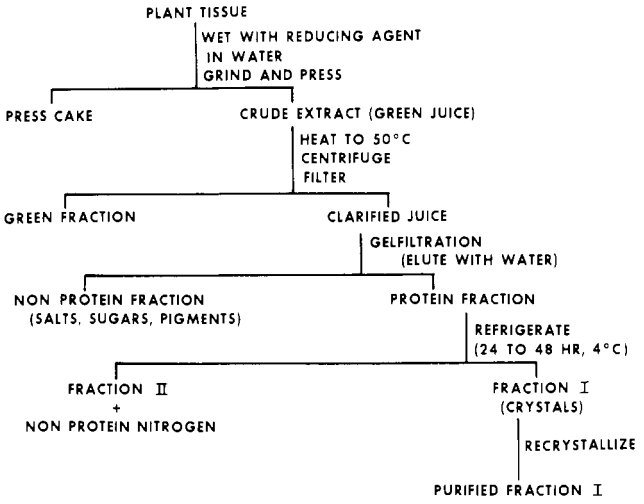

Figure 1. Scheme for processing tobacco for crystalline fraction I protein.

in ruminant feeds.

Pilot plant and commercial processes developed for the preparation of whole leaf protein concentrates (LPC) have been described in the literature (Hollo and Koch, 1970; Kohler et al., 1968; Pirie, 1971). Whole LPC is not readily accepted as a food because of its dark-green color and its undesirable flavor. By wet fractionation, a bland off-white LPC can be prepared from alfalfa (Bickoff and Kohler, 1974; Edwards et al., 1975; Knuckles et al., 1975; Kohler and Knuckles, 1977).

The predominant protein in leaf tissue is fraction I protein or ribulose-1,5-diphosphate carboxylase (EC 4.1.1.39) (Kawashima and Wildman, 1970, 1971). In the laboratory, this protein can be isolated from tobacco as pure white tasteless crystals which retain most of their native functional properties (Chan et al., 1972; Lowe, 1977). Before commercial production of fraction I protein for food or medical purposes is considered, nutritional and pharmacological tests are needed. These tests require relatively large quantities of materials which would be difficult to produce by present methods. This paper reports the development of a procedure in which 45-kg batches of tobacco can be processed to yield pure fraction I protein and other protein concentrates which may be useful as food or feed. The amino acid composition and nitrogen solubility of these fractions are given.

MATERIALS AND METHODS

Raw Material. The tobacco (*Nicotiana tabacum*, 'Turkish Samsun') was grown in a greenhouse at the University of California at Los Angeles (UCLA). In general, the plants were harvested at the age of 8–10 weeks, packed in ice in insulated containers, and shipped to the Western Regional Research Laboratory (WRRL) where the experiments were conducted. The period between harvest and experimentation was 24–48 h. The leaf fraction in these studies included the petioles. Both laboratory and large scale procedures followed, with minor differences, the processing scheme in Figure 1.

Laboratory Procedures. The laboratory test procedure was based on the previously reported method for preparing crystalline fraction I protein in the laboratory (Lowe, 1977). Briefly, this method involves extracting in a high salt medium containing a reducing agent, rupturing cells in a blender, squeezing the dark-green juice through cheese cloth, heating at 40 °C for 10 min, centrifuging to separate the chloroplastic particles, and desalting the soluble proteins by passing through a gel filtration column. The minor modifications that were made at this laboratory simplified and improved the procedure: (1) the plant tissue was wetted with reducing agent in water or buffered salt solution; (2) the tissue was ground and pressed in a Norwalk triturator-hydraulic press (Norwalk Foundry and Machine Co.); (3) the green juice was heated to 50 °C for 5 min; and (4) the protein solution was desalted by passing it through molecular sieve gels in columns of 2.5 × 35 cm or 5 × 50 cm. Several gels were investigated for use in preparing the crystalline fraction I protein. They included Sephadex G-25 and G-50 (Pharmacia Fine Chemicals) and Bio-Gel P-6, P-10, and P-30 (Bio-Rad Laboratories). Changes were made in reducing agent and in extraction and gel filtration steps as required by experimental design.

Large-Scale Procedure. Tobacco (45.3 kg) was wetted by dipping in 9 kg of water containing 130 mL of mercaptoethanol; the excess solution was allowed to drain off. The material was then ground in a disc type mill (Bauer Bros. Co., Model 148-8) having a disc clearance of 0.015 in., and the juice was pressed out by a single-screw press (Vincent Engineering Co., Model VP 6). The juice (50 kg) at pH 6.2 was pumped at a rate of 4.6 L/min through a steam injector where the temperature was raised to 50 °C. This temperature was maintained for 1 min by passing the heated juice through an insulated holding tube (1 in. × 33 ft). The juice was then cooled to 25 °C by passing it through a plate heat exchanger (Creamery Package Co., Model SC-3196) and its pH was raised to 7.4 by addition of Tris buffer solution. The green fraction was removed from the juice by pumping it through a high-speed solids discharging centrifuge, RCF max (relative centrifugal force) = 14500*g* (DeLaval Separator Co., Model BRPX-2075), operated in a batch-type mode. The supernatant, which contained a small amount of suspended green particles, was mixed with 1 kg of Celite 505 and filtered through a small horizontal filter (325 cm^2; Sparkler Manufacturing Co.). The clear filtrate was subjected immediately to gel filtration on Sephadex G-50 using water as the eluant. Four columns having a total volume of 92 L were used. Two columns had dimensions of 15.2 × 130 cm each and the other two columns had dimensions of 15.2 × 60 cm and 22 × 90 cm, respectively. The clear filtrate was added to the columns in batches equal to 18.5% of the bed volume. The effluent from the column was monitored for protein by adding small aliquots to equal volumes of 20% trichloroacetic acid (Cl_3CCOOH). The effluent fractions which contained protein were collected, pooled, and stored at 4 °C after making the solution 0.5 mM in $MgCl_2$ and 5 mM in $NaHCO_3$. The crystals which formed in 48 h were removed by centrifugation, using a continuous feed solid bowl centrifuge RCF max = 8940*g* (Gyrotester, DeLaval Separatory Co.). The crystals (320 g) were then dissolved in 0.2 M NaCl (1200 mL), and a small amount of insoluble material was removed by centrifugation at 48000*g*. The colorless solution was placed in dialysis tubing (Visking Co., size 3.25 in. × 20 in.) and dialyzed against Tris buffer (0.025 M Tris, 0.002 M EDTA, pH 7.4) for 48 h at 4 °C. The crystals were collected in a refrigerated centrifuge (Sorvall, Model RC 2B). Portions of the crystals were freeze-dried and spray-dried as described below. Other portions were dissolved in salt solution (0.1 M NaCl) at pH 7.4 and 8.5 and then dried. The supernatant solution from the first crystallization, containing most of the fraction II protein, was also spray-dried as described below. This spray-dried supernatant material (21.5 g) was suspended in water (300 mL), heated to 85–90 °C for 5 min, and then cooled to 25–30 °C. The insoluble material, largely fraction II protein, was collected by centrifugation,

water washed, and freeze-dried. This fraction II protein is not a single protein but a mixture of the other soluble proteins.

Spray Drying. The solution or suspension was pumped into a small conical type spray drier (Bowen Engineering Co., laboratory model). The inlet temperature of the dryer was controlled at 425–450 °F. The pumping rate (approximately 35 L/h) was adjusted to maintain an exit temperature of 220–225 °F.

Analytical Methods. Amino acid analyses were performed as described by Kohler and Palter (1967). Ether extractives, crude fiber, nitrogen, and ash content were determined by standard AOAC methods (AOAC, 1975). Crude protein was calculated as Kjeldahl N × 6.25. Nonprotein nitrogen (NPN) was assumed to be the nitrogen soluble in 10% Cl_3CCOOH. Solubility of nitrogen in the protein fractions was determined by extracting a 0.25-g sample three times with 20 mL of water and measuring the nitrogen in the combined extracts as well as in the insoluble material. The moisture content was calculated from sample weight before and after drying at 110 °C for 2 h in a forced draft oven.

RESULTS AND DISCUSSION

Preliminary Experiments. In the preparation of protein from plant materials, care must be taken to minimize browning reactions which can inhibit crystallization or enzymatic activity or which will result in a dark-colored product. Enzymatic browning occurs when proteins or peptides react with oxidized phenolic compounds (Smith and Johnson, 1948; Pierpoint, 1969a,b). Several reducing agents (ascorbic acid, sodium bisulfite, mercaptoethanol) have been used to inhibit the enzymes which cause darkening (Schroeter, 1966; Haisman, 1974; Anderson and Rowan, 1967; Loomis and Battaile, 1966; Bickoff and Kohler, 1974; Edwards et al., 1975). In addition to these compounds, sodium dithionite, sodium phosphite, sodium hypophosphite, and stannous chloride were evaluated for use in the preparation of fraction I protein. Most of these chemicals reduced the color in the clear protein solution that was applied to gel columns. However, in only three cases (ascorbic acid, mercaptoethanol, and sodium dithionite) were crystals obtained from the column effluent. When sodium metabisulfite was used a white noncrystalline protein precipitate formed. However, when Lowe (1977) used sodium metabisulfite in processing tobacco, crystalline fraction I protein was obtained.

In previously reported methods for the extraction of fraction I protein from tobacco, cell rupture occurred in the presence of a buffer with high salt content (Chan et al., 1972; Lowe, 1977). In preliminary experiments, we found that a high salt content was not necessary to obtain good yields of crystalline fraction I protein. In a typical experiment, the yield of crystalline fraction I protein was 4.02 g/100 g of dry leaf tissue when the leaves were wetted with water and 4.09 g/100 g of dry leaf tissue when the leaves were wetted with a 2.5 M NaCl solution.

Gel filtration was used to separate the protein fraction from salts and other factors that prevent crystallization of fraction I protein (Chan et al., 1972). The yield of crystalline fraction I protein was not dependent upon a particular gel (Sephadex or Bio-Gel), provided a good separation between the protein and pigment zones was achieved. Sephadex G-50 provided better separation of these zones than other gels, so it was selected for use in the large-scale process.

The yield of crystalline fraction I protein was affected by the type of starting material and the postharvest treatment. The yield of fraction I was >3% of the dry weight of tobacco leaves, but only 0.02% of the dry weight of the tobacco stems (Table I). Fraction I yields were also affected by the temperature of the tobacco during shipment from UCLA to WRRL. When the tobacco was shipped in a chilled condition, the fraction I yield from leaves (2.94 g/kg of fresh tissue) was similar to that obtained routinely from freshly harvested leaves at UCLA. When the tobacco was not chilled, the yields were 25–30% lower. The yield of fraction I from chilled leaves is greater than the yield obtained by Chan et al., (1972) (1 g/kg of fresh tissue) but less than the maximum yield reported by Lowe (1977) (6 g/kg of fresh tissue).

Table I. Yield of Crystalline Fraction I Protein from Tobacco Leaves and Stems

process and material	initial temp, °C	fraction I yield[a] g/kg of fresh tissue	fraction I yield[a] g/100 g of dry tissue
laboratory			
leaves	8-10	2.94	4.05
leaves	25	2.15	3.31
leaves + stems (50/50)	25	1.36	1.54
stems	25	0.02	0.019
large scale			
leaves	5	2.13	3.10
whole plant[b]	5	1.11	1.55

[a] Except for large-scale values, values are averages of two trials. [b] Ratio of leaf to stem was 2:1.

Table II. Laboratory and Large-Scale Extraction of Tobacco Components

component	laboratory values[a]	large-scale values[b]
juice yield,[c] %	82.3	80.1
dry matter extracted,[d] %	50.1	51.1
crude protein extracted,[e] %	54.6	57.3

[a] Average of three trials. [b] Single values. [c] As is basis, based on weight of fresh tobacco leaves processed. [d] Dry basis, based on total solids in leaves processed. [e] Based on crude protein content of tobacco leaves fed to grinder.

Large-Scale Experiments. Continuous grinding and pressing was an effective method of expressing a protein-rich juice from tobacco. The juice yield and extractability of dry matter and crude protein compare favorably with the values obtained in the laboratory (Table II).

The removal of the green chloroplastic material from plant extracts was accomplished by centrifugation. The relative centrifugal force required to sediment this fraction from extracts of chilled tobacco leaves was 48000*g* for 20 min. Since no continuous centrifuge can duplicate these conditions, the use of a mild heat pretreatment was investigated. Mild heat treatments have been used by others to aid in the separation of the green fraction from leaf extracts (Bickoff and Kohler, 1974; deFremery et al., 1973; Edwards et al., 1975; Henry and Ford, 1965; Lowe, 1977). According to Bahr et al. (1977), mild heat treatments (<60 °C for 5 min) do not cause the precipitation of fraction I protein from tobacco extracts. In our experiments, heating the plant juice at 50 °C for 1 min caused sufficient agglomeration of the chloroplastic particles so that almost all of this material could be removed by continuous centrifugation. This green fraction, which had a 19.6% dry matter content, represented about 8% of the dry matter in the starting material.

In order to prevent fouling of the gel columns and contamination of the protein solution, final traces of green

Table III. Composition of Process Fractions from Tobacco[a]

fraction	total solids,[b] %	crude protein,[c] %	fat, %	fiber, %	ash, %
whole plant	7.11	36.4	1.64	17.01	13.88
stem	10.62	23.8	3.02	31.00	12.73
leaf	6.84	52.4	6.21	10.02	13.31
process fractions from leaves					
press cake	29.58	26.7	7.03	22.6	8.25
crude extract	2.94	58.7	8.12	1.04	21.87
green fraction	19.60	46.1	14.13	4.47	18.74
clarified extract	2.45	51.4	2.77	0.92	24.50
fraction I		102.3	3.56	0.71	2.15
fraction II[d]		97.4	0.29	0.06	1.42
solubles fraction[e]		64.4	0.21	0.28	8.61

[a] Dry weight basis except where indicated. [b] As is basis. [c] N × 6.25, see text. [d] Insoluble material upon heating (85 °C) a suspension of spray-dried material remaining after removal of fraction I protein. [e] Soluble material upon heating (85 °C) a suspension of spray-dried material remaining after removal of fraction I protein.

material were removed by filtration with diatomaceous earth. The filtered solutions were clear and light brownish yellow. The color of the solution tended to darken during filtration. The darkening became severe if oxygen was not excluded from the system. It is possible to obtain light straw colored solutions from tobacco without the use of reducing agents when all processing is done in a nitrogen atmosphere (Cohen et al., 1956).

Salts and other inhibitors of fraction I crystallization are separated from the proteins by gel filtration. Satisfactory removal of these components was achieved if loading of the clarified solution on the column did not exceed 18.5% of bed volume. Large-scale gel filtration was limited to about 32 L of clarified solution per day. This was accomplished by making two runs per column with each run requiring about 2 h. It should be possible to process this quantity of clarified solution in 1 h if larger columns, similar to those designed by Janson (1971), are used.

Difficulty was encountered in recovery and recrystallization of fraction I protein on a large scale. Some of the equipment used was not designed for large-scale processing. The most difficult step was the removal of salt during recrystallization. Dialysis in Visking tubing was a batch process and limited the amount that could be recrystallized. Freeze-drying or spray-drying of the recrystallized fraction I protein resulted in a white powdered product.

Large-scale yields of fraction I crystals were lower than the yields obtained in the laboratory (Table I). About 95 g of recrystallized fraction I protein was obtained when 45 kg of leaves were processed. This was about twice the yield obtained from the whole plant.

Composition of Process Fractions. The composition of process fractions is given in Table III. The leaves, on a dry basis, were more than 50% crude protein and had a low fiber content. The crude extract (green juice) had 58.7% crude protein, a value which is considerably higher than the 35% crude protein content in juice from alfalfa (Edwards et al., 1977). About 40% of the nitrogen in this crude extract was actually NPN. In alfalfa juice, the NPN is 27 to 40% of the total nitrogen depending upon time, temperature, and pH while processing (de Fremery et al., 1973). Most of this NPN was separated from the protein during gel filtration. The NPN in the protein fraction from the column was only 6% of the total nitrogen present. The nitrogen content of the fraction I protein before and after recrystallization was 16.37 and 17.01%, respectively. If the conventional protein factor of 6.25 is used to calculate the protein content in these samples of fraction I protein, the values are greater than 100%.

Protein factors may be calculated from total nitrogen content or the amino acid nitrogen recovered during analysis. The factors calculated from total nitrogen content for fraction I and II proteins were 5.88 and 6.42, respectively. The protein factors for fraction I and II protein were 5.79 and 6.00, respectively, when the amino acid recoveries (Table IV) were used for calculation.

Table IV. Amino Acid Composition of Tobacco Protein Fractions[a]

amino acid	protein fraction: green[b]	fraction I[c]	fraction II[d]
alanine	5.62	6.13	6.44
(ammonia)	1.04	1.06	.70
arginine	5.12	8.06	7.00
aspartic acid	8.72	9.21	10.50
cystine	.99	2.00	1.78
glutamic acid	10.23	12.24	12.66
glycine	5.13	5.58	5.58
histidine	1.89	3.09	2.88
isoleucine	4.95	4.76	5.32
leucine	8.78	9.16	9.64
lysine	5.69	6.25	7.16
methionine	2.27	2.13	2.50
phenylalanine	5.72	5.68	5.81
proline	4.36	4.74	4.87
serine	4.62	3.18	4.37
threonine	4.28	5.37	5.12
tyrosine	3.62	6.58	6.03
valine	5.90	7.48	7.62
% N recov.	79.80	94.13	93.74
protein factor	5.89	5.79	6.00

[a] Grams amino acid per 16 g of nitrogen. [b] Mean for four samples. [c] Mean for four samples. [d] Insoluble material upon heating (85 °C) a suspension of spray-dried material remaining after removal of fraction I protein.

The amino acid composition of the protein fractions is given in Table IV. Except for cystine and methionine, the amino acid values for the green fraction protein were lower than the values reported for a similar fraction from alfalfa (Bickoff et al., 1975). When calculated on the same basis, the amino acid values for fraction I protein were similar to values reported earlier (Kawashima and Wildman, 1970). Based on the provisional amino acid pattern (FAO, 1973), both fraction I and II protein contain sufficient amounts of essential amino acids in the proper balance to serve as a high-quality food. Of particular note is the high lysine content in the fraction II protein.

Solubility of Nitrogen in Protein Fractions. Solutions and suspensions of fraction I protein, which had been spray-dried or freeze-dried, were tested for nitrogen solubility in water (Table V). Fractions dried at pH 7.4 had low solubility (5–12%). Salt did not appreciably increase the solubility of the dried protein. Fraction I protein had greater nitrogen solubility when it was dis-

Table V. Solubilization of Nitrogen in Protein Fractions with Water

material	pH	nitrogen solubility,[a] %
fraction I protein		
spray-dried[b]	7.4	5.05
freeze-dried[b]	7.4	12.81
fraction I protein (salt added)		
spray-dried	7.4	6.12
freeze-dried	7.4	12.64
freeze-dried	8.5	42.45

[a] Average for two values. [b] Dried as suspension; other samples were dried as solution.

solved in salt solution at pH 8.5 before drying.

The studies reported in this paper show that tobacco can be processed on a large scale to yield crystalline fraction I protein and other protein concentrates suitable for feed or food. Improved equipment for collection and recrystallization of fraction I should enable even greater quantities of tobacco to be processed.

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Influence of Nitrogen Fertilization on Potato Discoloration in Relation to Chemical Composition. 2. Phenols and Ascorbic Acid

Nell Irene Mondy,* Robert Louis Koch, and Subhash Chandra

The effect of nitrogen fertilization on enzymatic darkening of Katahdin potatoes was examined with respect to phenolic and ascorbic acid content. Ammonium nitrate was applied at rates of 100, 150, 200, and 250 lb/acre. Enzymatic darkening and phenolic content increased significantly ($p < 0.01$) as the level of nitrogen increased. A significant positive correlation ($r = +0.97$) was found between phenolic content and enzymatic discoloration. Ascorbic acid increased significantly ($p < 0.01$) with increased nitrogen levels.

Previous work from our laboratory has indicated the importance of nitrogen fertilization and its effect on tuber discoloration with regard to lipids, potassium, and dry matter content of Katahdin potatoes (Mondy and Koch, 1978). Potatoes receiving higher amounts of nitrogen exhibited greater enzymatic discoloration, increased dry matter, and lowered lipid and potassium content than control tubers. A significant negative correlation ($r = -0.95$) was established between the degree of enzymatic discoloration and lipid content of potato tubers. Other important chemical constituents in the potato tuber related to enzymatic discoloration are phenols and ascorbic acid.

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