A Water-Soluble, Stable Green Pigment, Originating during Processing of Canned Brussels Sprouts Picked before the First Autumn Frosts

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Brussels sprouts, when picked before the first autumn frosts, give, upon heating in water at 115° C., a water-soluble, stable green pigment, which appears to result from the association of a water-soluble protein moiety containing small quantities of copper and zinc, and chlorophylls and their derivatives. Neither phosphorus nor sugars are present. Of the 16 amino acids found, threonine is the only one to appear as an *N*-terminal amino acid, in the pigment itself as well as in the protein moiety isolated from the whole. Dissolved in water, the pigment resists light, heat, and weak acids.

Brussels sprouts, axillary buds of *Brassica oleracea var. gemmifera,* are customarily never picked before the first autumn frosts, either for shipping fresh or for canning; their flavor is thought to be too sharp when they are picked at an earlier date.

Preparation for canning is carried out by inspecting, trimming, and washing the buds, which are then blanched a few minutes in boiling water, drained, put into cans, and covered with hot 2% brine, slightly acidified with citric acid (1%); the cans are closed, processed at 115° or 120° C., and finally cooled in running water.

When the above procedure is applied to Brussels sprouts picked after the first autumn frosts, the liquor surrounding the buds in the can shows a yellowish color; the liquor is, on the contrary, bright green when the buds are picked before the first frosts. Moreover, the color is not affected by light, heat, or weak acids.

This observation, made during an experimental pack, prompted a closer investigation of the phenomenon. Its study may perhaps contribute to the preservation of green color in canned vegetables.

The problem has been the object of many investigations; rarely, however, have similar observations been reported. Schanderl *et al.* (1965a, 1965b) mention noticing occasionally, in canned peas, following the usual change of color resulting from the transformation of chlorophylls a and b into pheophytins, a reversion to the green color after prolonged storage. They ascribe this to binding by the pheophytins of copper and zinc naturally present in the plant. Szöke (1963) points to a similar occurrence in canned green beans, and Decleire (1966) ascribes this type of greening to the presence of metals. Some authors mention protein-chlorophyll complexes. Kahn and Bannister (1965) describe one from spinach leaves, soluble in a 0.2% (wt./v.) water solution of Trilon X 100 (a wetting agent). Haidak *et al.* (1966) report the presence, in *Gonyaulax polyedra,* of a peridinin-chlorophyll-protein complex with a molecular weight of 38,000, soluble in water and hydrolyzed by the combined action of trypsin and chymotrypsin. Popov and Bakurdjieva (1965) observe that the heat and light stability of vegetable pigment—protein complexes in aqueous solution is enhanced by traces of copper and diminished by traces of nickel.

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EXPERIMENTAL

Preliminary Tests. The following points have been established: The greening of the brine is not connected with the material of the container, and takes place in glass jars as well as in tin plate cans. A thermal treatment of 30 minutes at 100° C. does not produce any greening; however, a strong green color appears after 30 minutes of heating at 115° C. Sprouts from the same plant, picked before and after the first frosts, give a strong green color in the first case, but none in the second. This observation has been confirmed three years in succession. Storage at −21° C. for 3 days does not prevent the greening of sprouts picked before the first frosts. The formation of the green color occurs with Brussels sprouts from widely different growing areas. In the same plant, the sprouts farthest away from the ground give the most intense greening, and in any one sprout the outer leaves give a stronger green color than the inner leaves.

The formation of the green color appears to depend simultaneously on the absence of frosts during the vegetation of the plant and on the 30-minute heating at 115° C., the usual commercial process for canned Brussels sprouts.

The green pigment present in the brine dyes neither cotton nor casein. The green liquor gives a positive biuret reaction. Ammonium sulfate precipitates a green flocculent material. By extraction of the green brine with ether, chloroform, or methyl ethyl ketone, the color is transferred to the organic solvent, where it rapidly fades away into a yellowish tinge under the influence of light or heat.

Preparation and Purification of the Pigment. To the green liquor from canned Brussels sprouts picked before the first frosts, ammonium sulfate is added so as to reach a 45% (wt./v.) concentration. After centrifuging at 1500 G for 30 minutes at 4° C., the supernatant is discarded and the precipitate dissolved in a minimum amount of water, salted out again with ammonium sulfate, centrifuged, and once more taken up in water. This solution is dialyzed at 2° C. against double-distilled water until the dialyzate no longer gives a positive reaction with Nessler's reagent. Finally, the

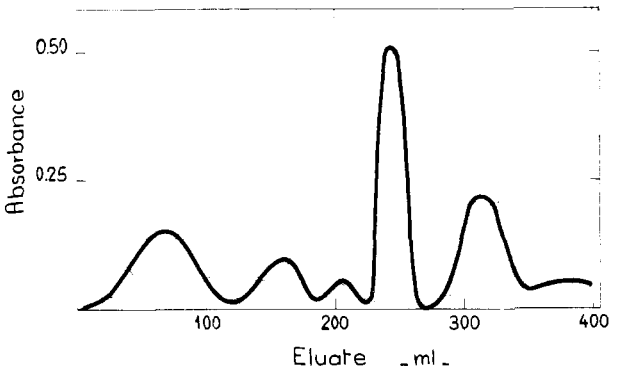

Fig. 1. Absorption of 260 mμ of eluate from Sephadex G 100

contents of the dialysis bag are freeze-dried. In this way, 10 liters of brine, corresponding to about 14 kg. of fresh sprouts, yielded 1.4 grams of green powder.

Five hundred milligrams of this powder are dissolved in 5 ml. of water, and the solution is sent through a column of Sephadex G 100 in double-distilled water (column diameter, 28 mm.; height, 800 mm.). The subsequent elution is done with double-distilled water (9 ml. per hour), and the eluate is collected in 10-ml. fractions, whose spectrophotometric absorption at 260 mμ is then recorded (Tristram, 1949). Figure 1 shows the presence of five fractions, three of which are colorless, one pale greenish-yellow, and one intensely bright green. Collected and freeze-dried, the latter fraction yielded 230 mg. of pigment; this material was used for the analytical work.

Analytical Procedures. Total nitrogen was determined by the method of Kjeldahl (Kirk, 1950); phosphorus with ammonium molybdate, according to Zinzadze (1935); copper with diethyldithiocarbamate (A.O.A.C., 1965); zinc with zincon (Yuan and Fiskell, 1958). Analysis for sugars was carried out by paper chromatography (Jermyn and Isherwood, 1949); amino acids were identified by paper chromatography (Woiwod and Linggood, 1949); *N*-terminal amino acids were determined with DNP by the procedure of Chauvet *et al.* (1966); chlorophylls, chlorophyllides, and pheophytins were evaluated spectrophotometrically according to White *et al.* (1963).

RESULTS

Nitrogen, Phosphorus, Sugars. The total nitrogen content of the dry green pigment amounts to 6.9%; 91.3% of the total nitrogen remains in the colorless aqueous phase obtained by dissolving the pigment in water and extracting with methyl ethyl ketone. No phosphorus appears to be present (<1 mg. per kg.); no sugars were found, either before or after hydrolysis (2*M* HCl, 30 minutes, 100° C.).

Copper and Zinc. The presence of copper (18 ± 4 mg. per kg.) and zinc (12 ± 2 mg. per kg.), and the possible role of these metals with respect to the green color and its stability, prompted an effort to eliminate them. A solution containing 50 mg. of pigment in 50 ml. of water was dialyzed 24 hours at 2° C., against an aqueous 3% solution of complexone III. After freeze-drying, the residue contained the same levels of copper and zinc as before dialysis. A dialysis conducted

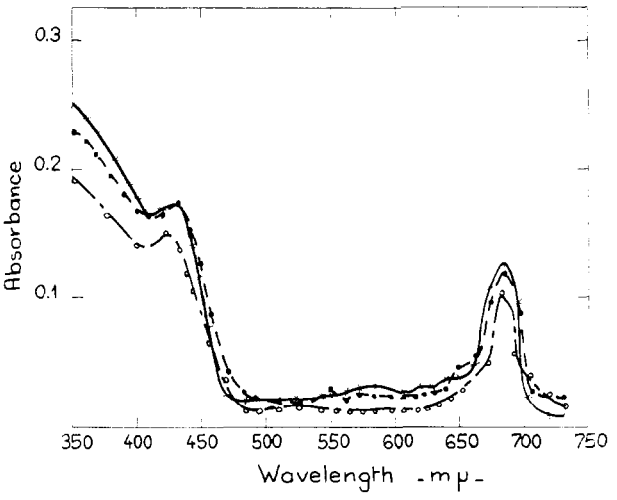

Figure 2. Absorption spectrum of the pigment in water

× ——— original solution
● - - - - after trypsin attack
○ — - — after trypsin attack and dialysis

in the same way, but against double-distilled water and after adding 100 mg. of complexone III to the solution in the bag, led to the same result. In another attempt, a solution of 500 mg. of pigment in 20 ml. of water was extracted with methyl ethyl ketone until the green color was removed from the aqueous phase. All copper and zinc initially present were found in the aqueous phase. Dialysis of the latter for 24 hours at 2° C. against a 3% solution of complexone III failed to remove any copper or zinc.

Amino Acids. Paper chromatography of 20 mg. of pigment in 5 ml. of water, after hydrolysis in a sealed tube (6*M* HCl, 18 hours, 100° C.), showed the following amino acids to be present: aspartic acid, glutamic acid, histidine, lysine, arginine, leucine, glycine, α-alanine, β-alanine, valine, proline, cysteine, methionine, serine, threonine, tyrosine. The same 16 amino acids were found in the hydrolysate of the colorless aqueous phase remaining after extraction with methyl ethyl ketone.

***N*-Terminal Amino Acids.** Since the results so far obtained indicated that some protein-like material was present as a constituent of the pigment, an attempt was made to identify its free *N*-terminal amino acids. This was done on the pigment itself as well as on its aqueous solution after removal of the green component by extraction with methyl ethyl ketone. In both cases, one single amino acid was found: threonine.

Action of Trypsin. A solution of 20 mg. of pigment in 20 ml. of water, brought to pH 8.0 with 0.1*M* ammonium carbonate, was kept 24 hours at 37° C. after adding 1 mg. of crystallized trypsin (Merck), and dialyzed for 36 hours at 2° C. against double-distilled water. Figure 2 gives the absorption spectra, between 350 and 700 mμ, of the initial solution, the solution after trypsin attack and the solution after trypsin attack and dialysis. The three spectra are similar, except for the fact that the one given by the dialyzate is slightly lower than the two others.

Absorption Spectrum in Visible Light. This was determined on the ether extract of an aqueous solution of the pigment. The curve (Figure 3) shows the following

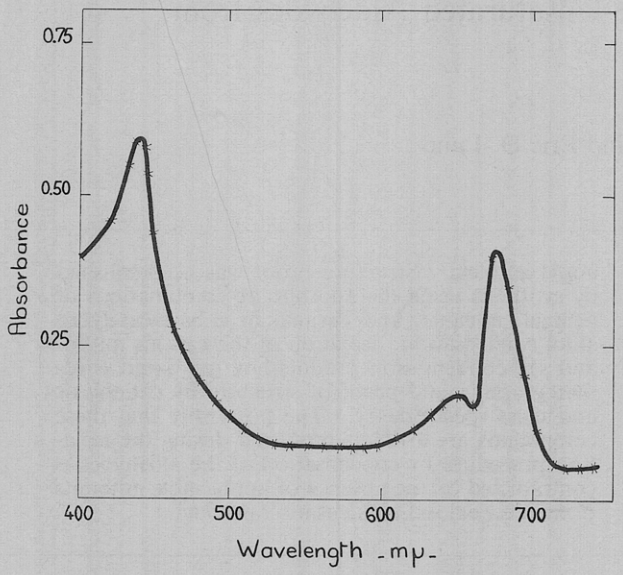

Figure 3. Absorption spectrum of the pigment in ether

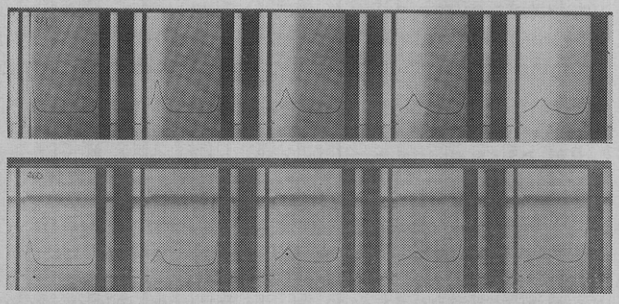

Figure 4. Ultracentrifugal pattern of the green pigment (top —20 mg./liter) and the protein moiety (bottom—27 mg./liter) in double-distilled water

Spinco Analytical Type E ultracentrifuge; 259,700 G max. at center of cell; 20° C.; time interval between photographs, 32 minutes

maxima, placed beside those of chlorophylls a and b for comparison:

Ether extract (mμ)	Chlorophyll a (mμ)	Chlorophyll b (mμ)
430	430	
		453
644		641
662	660	

Tentative Evaluation of the Substances Extracted by the Organic Solvents. Assuming the compounds in the extract to be chlorophylls and chlorophyll derivatives, the procedure described by White *et al.* (1963) leads to the following figures (as per cent of pigment): chlorophyll a, 16.7; chlorophyll b, 2.4; chlorophyllide a, 2.9; chlorophyllide b, 10.6; pheophytin a, 5.1; pheophytin b, 0.3.

Sedimentation Constants. Figure 4 gives the ultracentrifugal patterns of the pigment and its protein moiety. Each preparation appears to contain at least two components, which sediment at almost the same speed.

The sedimentation constants, $s_{20,w}$, calculated from the main peak of each pattern, are the following: pigment, 2.0×10^{-13}; protein moiety, 2.6×10^{-13}.

From these data it may be tentatively estimated that the molecular weight of the pigment lies in the range 15,000 to 45,000.

CONCLUSION

The pigment appears to be a complex of a water-soluble protein moiety carrying small amounts of copper and zinc, and chlorophylls and their derivatives.

The rather peculiar circumstances necessary for its formation deserve further study. The identification of the precursors and the role of temperature both upon the plant and upon the reaction leading to the pigment should retain the attention of plant biochemists.

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