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CHLOROPHYLL DEGRADATION PRODUCTS FROM PROCESSED PEA PURÉE

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Abstract—In addition to chlorophylls *a* and *b* and pheophytins *a* and *b*, three chlorophyll degradation products were found in pH-elevated, high temperature–short time processed pea purée after storage for 18 months at room temperature. The pigments, separated and purified by cellulose TLC, are shown to have chemical and spectral properties similar to various alteration products of pheophytins *a* and *b* and chlorophyll *b*. Possible structures of the three pigments are discussed.

INTRODUCTION

CONSIDERABLE attention has been focused on methods to prevent or diminish the adverse colour change that occurs in green vegetables during heat processing and post-process storage. Such methods have centred on high temperature–short time (H.T.S.T.) processing[1–3] in combination with pH elevation[4–6] as a means of obtaining high-quality products with a minimum of colour deterioration. Products have been produced which are superior to conventionally processed materials in terms of colour and flavour, but evidence is lacking concerning their long-term storage stability.

In an investigation of the effect of storage time and temperature on the chlorophyll pigment composition of H.T.S.T.-processed pea purée, unusual spectral curves were observed in total pigment extracts of pH-elevated puree stored up to 18 months at room temperature. This paper describes the isolation of the pigments responsible, and their possible structures are discussed in relation to the properties of previously described chlorophyll degradation products.

RESULTS

During an extensive study of the storage behaviour of pH-elevated, H.T.S.T.-processed pea purée, it became apparent that chlorophyll pigment concentrations determined by spectrophotometry on total pigment extracts[7,8] were in error because of the presence of a pigment or several pigments with spectra different from those of the chlorophyll pigments normally found in heat-processed foods, i.e. chlorophylls *a* and *b* and pheophytins *a* and *b*.

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The calculated concentration of chlorophyll *b* in pea purée processed at 149° decreased progressively during storage for 3 months at 20° but then increased from 16·8 μg/g to 22·4 μg/g up to 18 months' storage. Chlorophyll *a* content also appeared to increase during storage from 12 to 18 months. The ratio of total *a* to total *b* pigments changed from 2·51 at 6 months' to 1·50 at 18 months' storage.

A comparison of the spectra of ether solutions of total pigments from normal and pH-elevated purée showed the latter to have absorption maxima shifted towards the "blue" end of the spectrum.

Chromatography of Total Pigment Extracts

Cellulose TLC of pigments in ether from pH-elevated purée stored 18 months at 20° showed the presence of five pigments with chromatographic behaviour and absorption spectra different from the usual chlorophyll pigments present in heat-processed vegetables. In addition to the chlorophyll-derived pigments, several carotenoids and their breakdown products were found but were not studied in any detail.

The absorption maxima in ether and approximate R_f values of the separated chlorophyll pigments are shown in Table 1. Unidentified pigments are designated pigments X, Y and Z in order of increasing adsorption on cellulose.

TABLE 1. CHLOROPHYLL-DERIVED PIGMENTS ISOLATED FROM H.T.S.T.-PROCESSED pH-ELEVATED PEA PURÉE STORED 18 MONTHS AT 20°

Pigment	Colour on cellulose	Colour under u.v.	Principal absorption maxima in Et_2O (nm)	Approx. R_f on cellulose	Approx. % of total chlorophyll-like pigments
Pheophytin *a*	Grey	Dark red	666, 409	0·87	35
Pheophytin *b*	Yellow-green	Red	653, 434	0·75	15
Pigment X	Green-grey	Red	664, 401	0·70	30
Chlorophyll *a*	Blue-green	Red	660, 429	0·64	< 5
Pigment Y	Yellow-green	Red	653, 427	0·54	15
Chlorophyll *b*	Green	Red	642, 454	0·44	< 5
Pigment Z	Green	Orange-pink	630, 444	0·30	5
Pigment Z Derivative?	Faint green	Faint pink	630*, 446*	0·20	trace
Pigment X Derivative?	Not visible	Faint pink	661*, 400*	0·10	trace

* Absorption maxima in acetone.

TLC of extracts from normal pH purée stored 18 months at 20° showed pheophytins *a* and *b* to be the major chlorophyll-derived pigments, with only trace amounts of compounds corresponding to pigments X and Y. Chlorophylls *a* and *b* and pigment Z were not detected. TLC of extracts from normal and pH-elevated puree stored 18 months at −23° showed the presence of pheophytins *a* and *b*, chlorophylls *a* and *b* and trace amounts of chlororphylls *a'* and *b'*. Compounds corresponding to pigments X, Y and Z were not detected.

Properties of Pigments X, Y and Z

1. *Visible absorption spectra.* Typical spectra of pigments X, Y and Z in diethyl ether are shown in Fig. 1.

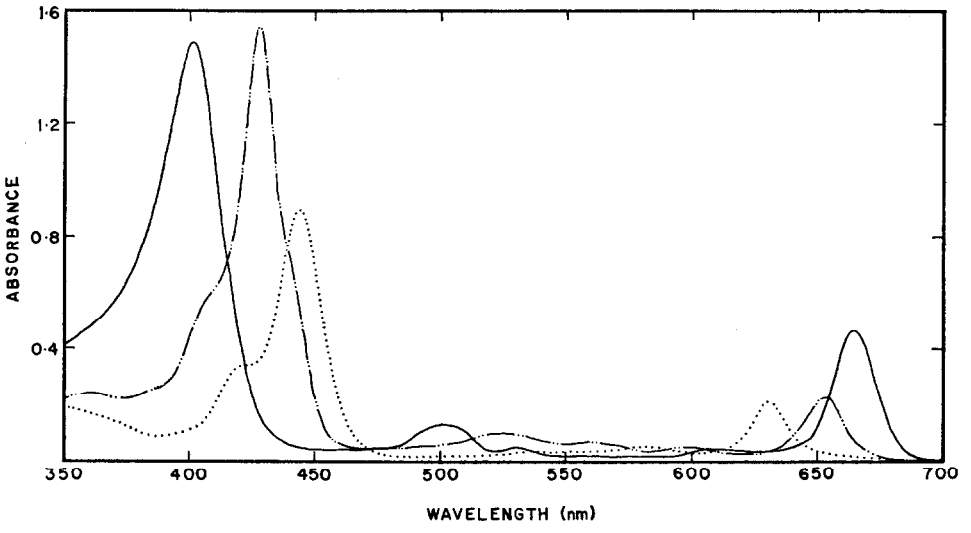

FIG. 1. ABSORPTION SPECTRA OF PIGMENT X (——), PIGMENT Y (-·-·-) AND PIGMENT Z (·····) IN DIETHYL ETHER.

Pigment X was present at a slightly lower concentration than pheophytin *a* and its spectrum appeared similar to pheophytin *a*. However, there were a number of important differences: both the "red" and "blue" maxima (664, 401 nm) were shifted to the "blue" compared to pheophytin *a* (666–667, 409 nm); the ratio of peak heights in the region 500–540 nm was significantly larger in pigment X than in pheophytin *a* because of the diminished height of the peak near 530 nm; the blue/red absorbance ratio was greater for pigment X (range 2·84–2·93 for different preparations) than pheophytin *a* (2·03–2·08). These differences corresponded closely to the discrepancies observed between the spectra of total pigment extracts from pH-elevated and normal purée.

The spectrum of pigment Y was similar to pheophytin *b*, although the blue absorption maximum (427 nm) was at a shorter wavelength than pheophytin *b* (434 nm). The secondary blue peak (412 nm) was reduced to an inflection at approximately 406 nm, and the blue/red absorbance ratio (6·82–6·92) was higher compared to pheophytin *b* (5·16–5·22).

The spectrum of pigment Z was similar to chlorophyll *b*, although both red and blue absorption maxima were shifted substantially towards the blue, and the blue/red absorbance ratio (4·44–4·51) was increased compared to chlorophyll *b* (2·81–2·84). Pigment Z displayed a distinctive orange-red fluorescence when viewed under u.v. light.

The two pigments most strongly adsorbed, with R_f 0·20 and 0·10, showed spectra similar to pigment Z and pigment X respectively, but were not present in sufficient concentration for further characterization.

2. *Magnesium-free derivatives.* The addition of HCl to acetone solutions of pigments X and Y did not change the absorption maxima of the pigments when measured in ether, although the blue/red absorbance ratios increased slightly. There was no colour change for either pigment. The acidification of pigment Z in acetone resulted in a colour change from green to yellow-green. Acid-treated pigment Z showed principal absorption maxima at 427 and 653 nm (ether), and a blue/red ratio of 6·95. When pigment Y and acid-treated (Mg-free)

pigment Z were co-chromatographed on cellulose, only one band was formed after development with Bacon's[9] Solvents A and B.

3. *Infrared spectra.* The i.r. spectra of concentrated solutions of pigments X and Y in CCl_4 showed similar absorption to pheophytins *a* and *b* throughout most of the wavenumber range with the exception of a substantial reduction in intensity of the major peak near 1708–1710 cm^{-1}. The prominent absorption near 1740 cm^{-1} was present in all four pigments. A spectrum could not be determined on pigment Z because of lack of material.

4. *Phase test.* Ether solutions of pigments were carefully underlayered with 30% (w/v) KOH in methanol.[10] Chlorophylls *a* and *b* and pheophytins *a* and *b* showed positive reactions, but pigments X, Y and Z all gave negative results.

5. *Partition between ether and hydrochloric acid.* Ether solutions of pigments were shaken with an equal volume of aqueous HCl, and the per cent by weight determined of HCl in solution which extracted more than two-thirds of the pigment from ether (=HCl number). The approximate HCl numbers of pigments X and Y were 23 and 27 respectively. Under the same conditions, the approximate HCl numbers of pheophytins *a* and *b* were 29 and 35 respectively, and of pheophorbides *a* and *b*, 15 and 20 respectively.

6. *Partition between ether and aqueous KOH.* When ether solutions of pigments X, Y and Z were shaken with an equal volume of 0·01 N KOH, the pigments remained in the ether phase, as did chlorophylls *a* and *b* and pheophytins *a* and *b*. Under the same conditions, pheophorbides *a* and *b* were extracted into the aqueous alkaline phase.

DISCUSSION

The presence of unusual chlorophyll-type pigments in heat-processed foods has not previously been reported in any detail. In an examination of the effects of H.T.S.T. processing and pH adjustment on chlorophyll retention in spinach purée, Gupte[11] observed that total pigment concentrations in pH-elevated samples decreased markedly during storage for 6 months at room temperature. Previously, other workers[4] had noted that in pigment analyses on pH-adjusted spinach purée, the analytical data indicated the presence of more pheophytin than could be accounted for by the degradation of chlorophyll. It was concluded that some substance was formed during processing that had higher absorption coefficients than the pheophytins, and was probably present as a contaminant of one of the zones separated by column chromatography. It would appear that similar compounds have been isolated in the present investigation.

Pigments X, Y and Z were similar in many respects to chlorophyll degradation products reported recently in ripening peppers, banana peel and cucumber peel,[12–14] to degradation products from chlorophylls after chemical and physical treatments of leaves and leaf extracts,[15,16] to artifacts from chromatography of purified pigments on certain adsorbents,[17,18] and to pigments in extracts of *Chlorella*.[19]

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Pigment Z showed similar spectral and chemical properties to "changed" chlorophyll *b*-3,[17] methoxy- (or hydroxy-) lactone chlorophyll *b*,[20] and a 444 nm chlorophyll-type pigment isolated from tissues of ripening peppers.[13] Each of these pigments showed absorption maxima displaced considerably toward the "blue" compared to chlorophyll *b*, were phase test negative and were not extracted into dilute aqueous KOH. "Changed" chlorophyll *b*-3 has been postulated to have a lactone-type structure, and this appears reasonable in view of its similarity to 10-methoxy (-hydroxy) lactone chlorophyll *b*,[20] and its chemical and chromatographic behaviour. Identification of pigment Z in terms of this structure is difficult in view of the differences between Mg-free pigment Z, which shows almost identical spectral and TLC properties to pigment Y, and Mg-free "changed" chlorophyll *b*-3.[21] The latter pigment shows maxima in ether at 426 and 658 nm, with blue/red absorbance ratio 4·90, while pigment Y shows maxima at 427 and 653 nm and blue/red ratio approximately 6·9. Similarly pigment X shows a different "red" maximum and blue/red ratio compared to Mg-free "changed" chlorophyll *a*-3.

The absorption spectrum and properties of pigment X are similar to those described by Briat *et al.*,[22] for various metal-free substituted chlorins. They found that rupture of the cyclopentanone ring of methyl pheophorbide *a* to form derivatives of chlorin e_6, rhodochlorin and isochlorin e_4 led to hypsochromic shifts of absorption of both the blue and red peaks, with an increased blue/red absorbance ratio. In particular, the minor inflections below 410 nm, present in pheophytin *a* or pheophorbide *a*, were absent in the derivatives, and the absorption of the secondary maximum at approximately 530 nm was greatly diminished as in pigment X. However, the presence of a —COOH group at C-6 in a chlorin e_6 derivative would be expected to reduce the TLC mobility of the pigment, while an isochlorin e_4 derivative would require decarboxylation of the C-6 carboxyl group. The oxidation of pheophytin *a* or *b* to purpurin 7-derived compounds would also be untenable since these derivatives show absorption maxima in the red at wavelengths higher than 680 nm.[23]

The substantial reduction in intensity of the prominent i.r. absorption peak near 1710 cm^{-1} in pigments X and Y compared to pheophytins *a* and *b* would tend to indicate the loss of the cyclopentanone ring in the derived pigments. However, Holt[24] has shown that the 1710 cm^{-1} peak of "unstable" chlorin-methyl-phytyl ester occurs only as an inflection on the more prominent ester carbonyl peak near 1740 cm^{-1}. Consequently, pigment X, and possibly pigments Y and Z also, may contain a lactone configuration in ring V as well as other minor alterations to the molecule giving rise to spectral and chromatographic differences.

Incubation of pheophytins *a* and *b* and chlorophyll *b* with sodium bicarbonate and magnesium carbonate in 50% aqueous acetone produced a number of degradation products separable by cellulose TLC. Two pigments isolated from incubated pheophytin *a* showed spectra and TLC behaviour similar to pigment X. The major degradation product, with maxima near 400 and 670 nm in diethyl ether, appeared similar to Mg-free "changed" chlorophyll *a*-3.[21] Another derived pigment with maxima at 400 and 665 nm and blue/red ratio 3·16 gave a spectral pattern near 500–530 nm very similar to pigment X, but showed a lower R_f and high absorption near 690 nm. The isolation of a pigment with properties and

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spectral maxima very similar to the "changed" chlorophyll derivatives, but unlike pigment X, is further evidence to indicate pigments X, Y and Z probably contain more than an —OH group at C-10 and/or a lactone configuration in the cyclopentanone ring.

The major degradation products derived from the incubation of pheophytin *b* with sodium bicarbonate and magnesium carbonate showed absorption maxima similar to pigment Y, but with lower blue/red absorbance ratios and slightly higher R_fs. Pigments derived from incubated chlorophyll *b* also showed similar absorption maxima to pigment Z but higher R_f values.

Although the observed differences between pigments X, Y and Z and other chlorophyll degradation products were considered significant under the conditions used in the present work, nevertheless their overall similarity indicates only minor structural differences. Further work on the identification of these degradation products is continuing. The absence of an *a*-series compound corresponding to pigment Z could be accounted for by the more rapid conversion of such a pigment to the Mg-free derivative, i.e. pigment X.

EXPERIMENTAL

Materials

Pea purée. Fresh green peas (*Pisum sativum*, var. Edgell Freezer) were shelled, blanched 1 min in boiling water, cooled, blended with distilled water, then comminuted under N_2 to form a purée of pH 6·95 and moisture content 86–86·5 per cent (=normal pH purée). Pea purée of pH 8·45 was prepared by blending blanched peas and distilled water with magnesium carbonate powder followed by comminution.

Deaerated purée was filled into Pyrex glass thermal death time tubes (25 cm × 0·7 cm i.d., walls 0·1 cm thick), which were sealed and then processed for 68 sec in glycerol at 149° to a process value of $F_0 = 6{\cdot}0$, based on Z=18 for *Clostridium botulinum* spores using extrapolated thermal death time data.[25] The maximum temperature in the centre of the TDT tubes was 136°. The tubes were immediately cooled in ice water, and stored at 20° and −23° for 18 months.

Solvents. Anaesthetic grade diethyl ether was purified for spectrophotometry by repeated distillation, treatment with acidified $FeSO_4$ solution and thorough drying.[26] Other solvents used were A.R. grade or spectroscopic grade.

Methods

Thin-layer chromatography. Ether solutions of pigments were chromatographed in the dark at room temperature on thin layers of cellulose powder (Whatman CC41, 0·5 mm) after the methods of Bacon[9] and Bacon and Holden.[15] Pigments were applied as a streak using the apparatus of Monteiro.[27] The adsorbent edges at right angles to the pigment streak were scraped away with a razor blade to prevent "side effects" during development. The chromatograms were developed in the dark with solvent A or solvent B[15] for a distance of 15 cm in glass tanks previously equilibrated with solvent for 30 min. Pigment bands were detected and marked under daylight or u.v., and scraped off the plate into a sintered funnel, and pigments eluted with acetone or ether for spectral analysis.

Column chromatography. Chlorophyll pigment fractions were rechromatographed using cellulose TLC, or on wet-packed columns of 10% (w/v) cellulose powder (Whatman CF11) in powdered icing sugar (containing 5% starch) and developed with 0–1% (v/v) acetone in petroleum ether (b.p. 60°–80°).

Model systems. Pheophytins *a* and *b* and chlorophyll *b*, purified on sugar/cellulose columns and by cellulose TLC, were incubated 4 days in the dark in M/100 $NaHCO_3$ or $MgCO_3$ in 50% (v/v) acetone. The pigments were transferred to ether, dried with anhydrous Na_2SO_4, and chromatographed using cellulose TLC with solvents A or B. The separated pigment bands were scraped off the plates, the pigments eluted with ether, and their spectra recorded.

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