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Comparative degradation pathways of malvidin 3,5-diglucoside after enzymatic and thermal treatments

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Some kinetic properties of an industrial anthocyanin-β-glucosidase (anthocyanase), Pectinol DL, with malvidin 3-monoglucoside and malvidin 3,5-diglucoside (malvin) as substrates, have been studied at pH 3 and 6. Pectinol DL is more active at pH 3 than at pH 6, the enzyme affinity being greater with malvidin 3-monoglucoside as a substrate (K_m = 0.72 mM) than with malvin (K_m = 3.2 mM). After hydrolysis of *O*-glucosidic bonds of malvin by Pectinol DL, the aglycone was very quickly destroyed. By TLC, GC and MS analysis, syringic acid and 2,4,6-trihydroxybenzaldehyde were identified. The same breakdown products were found after heating an aqueous solution of malvin at pH 3. An additional product was identified as phloroglucinol. From these results, it can be postulated that the aglycone is degraded according to a chemical mechanism proceeding to a carbon–carbon bond breakdown from the chalcone form.

INTRODUCTION

Anthocyanins are naturally occurring pigments, which are severely damaged during the processing of fruit and berries. The effects of various factors on anthocyanin stability have been studied by several authors. Thermal stability has been studied by Markakis *et al.* (1957), Daravingas and Cain (1968), Adams (1973) and Abers and Wrolstad (1979).

It has been shown that photochemical degradation is an important factor in pigment breakdown (Jurd, 1969; Maccarone *et al.*, 1987).

Enzymes accountable for pigment decolorisation were characterised as β-glycosidases (Huang, 1955; Harborne, 1965; Blom, 1983) and polyphenoloxidases. Recently, Jiang *et al.* (1990) and Rommel *et al.* (1990) showed a β-glycosidase activity on raspberry anthocyanin in industrial pectinolytic preparations. Polyphenoloxidase has been studied respectively from strawberry and sweet cherry by Cash and Sistrunk (1971) and Pifferi and Cultrera (1974).

Very few studies have addressed the structural determination of breakdown products from anthocyanins. Adams (1973) proposed a mechanism of cyanidin 3-glycoside breakdown by thermal treatment, leading to α-diketone via chalcone. Powers *et al.* (1960) identified phloroglucinol when apigenidin chloride was heated. 2,4,6-Trihydroxybenzaldehyde was identified (Maccarone *et al.*, 1987) when pelargonidin 3,5-diglucoside and cyanidin were submitted to photochemical treatment. Finally, breaking of the *O*-glycosidic bond with aglycone liberation has been demonstrated by several authors (Blom & Thomassen, 1985; Eskin, 1979).

The present work was undertaken to identify the ultimate degradation products of malvin after comparative enzymatic (Pectinol DL, pH 3) and thermal treatments (100°C for 15 h, at pH 3, under anaerobic conditions).

This allows us to propose a mechanism for thermal and enzymatic degradations of anthocyanins under aqueous acidic conditions.

MATERIALS AND METHODS

β-Glucosidase source

The anthocyanin-β-glucosidase used was a pectinolytic enzyme preparation, named Pectinol DL (Genencor, USA). This preparation was made from a thermophilic strain of *Aspergillus niger* which had been already partly purified (1.3 mg ml^{-1} of protein).

Anthocyanins

Malvidin 3-monoglucoside and malvidin 3,5-diglucoside (malvin) were purchased from Extrasynthese, France.

Standard assay for anthocyanase activity

Enzyme activity was measured in McIlvain buffer at pH 3 and 6 at 25°C. The anthocyanin concentrations were in the range 0.275–0.825 mM for malvin and in the range 0.037–0.112 mM for malvidin 3-glucoside. The assays were carried out in 1 cm cuvettes at 525 nm using 65 μg of protein enzyme per ml of reaction volume.

At pH 3, the decolorisation was initiated by enzyme addition and read using a Shimadzu spectrophotometer, model UV 260, relative to a blank solution without anthocyanin. The change in absorbance could be recorded continually at a specific wavelength (525 nm) or every 3 min across a spectrum between 200 and 600 nm.

At pH 6, the decolorisation was not developed directly in the spectrophotometer cuvette. A 0.1 ml volume of the reaction medium was added to a cuvette containing 0.9 ml of 0.1 M buffer (pH 6) and 50 μl 8 M HCl. This lowered the pH to 1, stopping enzymatic activity; the absorbance at 525 nm was measured after standing for 1 h in the dark.

Standard assay for thermal breakdown of malvin

A malvin solution (20 mg in 20 ml of McIlvain Buffer, pH 3) was introduced into an assay tube hermetically sealed with a rubber cap pierced by two needles. The solution was maintained under nitrogen, bubbling for 10 min, and placed on a boiling-water bath (100°C) for 15 h.

Extraction of breakdown products

The malvin solution, discoloured by Pectinol DL overnight at 25°C, was acidified to pH 1 with 6 M HCl. Phenolic compounds were extracted with ethyl acetate of high-performance liquid chromatography grade. The solvent was evaporated and the residue dissolved in 5 ml of 5% aqueous Na_2CO_3 (pH 8). Neutral phenols were extracted from this Na_2CO_3 solution by ethyl acetate (3 × 10 ml) and concentrated to 1 ml by evaporation of the solvent. This extract was labelled 'phenols fraction' (PF). The aqueous phase was acidified to pH 1 with 6 M HCl, and the phenolic acids were extracted from it with ethyl acetate. After evaporation of the solvent, the extract obtained (1 ml) was labelled 'phenolic acids fraction' (PAF).

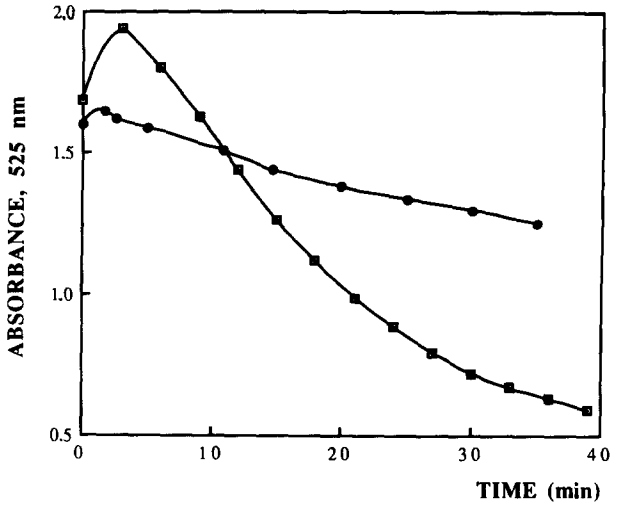

Fig. 1. Variation in the absorbance of malvidin 3,5-diglucoside at 525 nm, during its degradation by Pectinol DL at pH 3 (■) and at pH 6 (●), at 25°C.

Analytical methods

Anthocyanins were analysed by thin-layer chromatography (TLC) (DC-Alufolien Cellulose, 2 mm thick, Merck) with a water–hydrochloric acid–acetic acid mixture (8:4:1 v/v).

The phenolic compounds were located on TLC plates with diazotised 4-nitroaniline (Van Sumere *et al.*, 1965). Aldehydic groups were identified by 2,4-dinitrophenylhydrazine (DNPH) (Mehlitz *et al.*, 1963).

The breakdown products were identified by gas chromatography (IGC 121 FL Intersmat) coupled with mass spectrometry (Nermag/Sidar R-10 10-C).

A glass capillary column (25 m length, 0.32 mm i.d.) charged with SE-30 was used. The pressure of the carrier gas He was fixed to 11.76 psi at the head of the

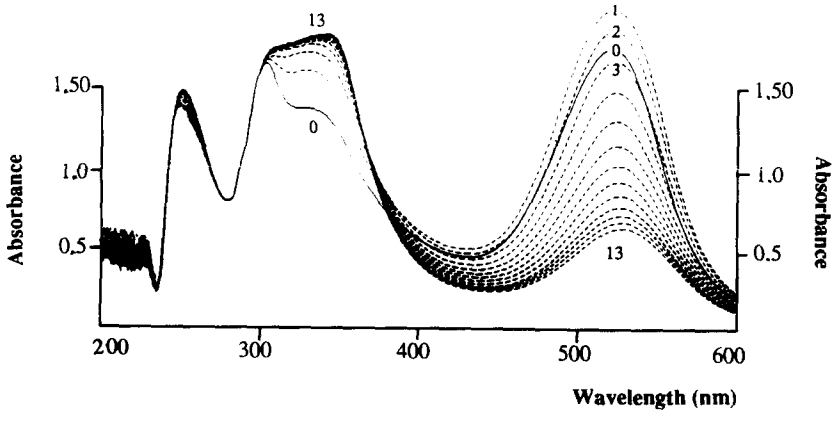

Fig. 2. Absorbance spectra (200–600 nm) of malvidin 3,5-diglucoside during its degradation by Pectinol DL at pH 3 and 25°C. Scan 0: malvin measured immediately. All scans were at intervals of 3 min except the interval between scans 12 and 13, which was 39 min.

column so that a flow of 2 ml min^{-1} of effluent was sent into the MS source. The injector and detector temperatures were set to 220°C. The column temperature was maintained at 150°C for 2 min and then monitored from 150 to 220°C (2°C min^{-1}). Injection volumes were 2 μl before the splitter (10% sent to MS). Mass spectrometry was used with electronic impact (EI, 70 ev) and chemical ionisation (CI, NH_3).

RESULTS AND DISCUSSION

The action of Pectinol DL is known to induce the hydrolysis of *O*-glucosidic bonds, having been studied at pH 3 and 6 on malvidin 3,5-diglucoside. At pH 3, malvin is sufficiently stabilised as flavylium ion to follow its degradation by spectrophotometric absorbance measurement at 525 nm. Although the optimal pH is 4.6 for Pectinol DL, according to the results presented by Huang (1955) and Blom (1983), enzymatic activity is great enough at pH 3.

At pH 6, the major form of malvin in aqueous solution is the carbinol form. In order to measure the enzymatic activity by spectrophotometry, it was necessary to acidify the reaction mixture to pH 1. As shown in Fig. 1, the Pectinol DL activity is greater at pH 3 than at pH 6. This implies two possibilities: either flavylium ion is a better substrate than carbinol for enzyme or enzymatic activity is higher at pH 3 than at pH 6 regardless of the anthocyanin structure.

However, although slower at pH 6, the enzymatic action still leads to complete decolorisation of malvin. Therefore, Pectinol DL may also act on the carbinol structure.

Malvin degradation at pH 3 is shown in Fig. 2 using 50 μl of Pectinol DL solution (65 μg of protein). It can be seen that the absorbance at 525 nm is increasing during the first 5 to 6 min. This increase proves the breakdown of glucosidic bonds (in order 3 and 5, or 5 and 3) with release of aglycone. Indeed, the molar extinction coefficient of malvidin is higher than that of the corresponding anthocyanins (Ribereau-Gayon, 1972). A concomitant increase in the absorbance at 330 nm and then a decrease at 525 nm can also be observed. The absorbance at 330 nm probably corresponds to chalcone formation after opening of the anthocyanin ring (Preston & Timberlake, 1981).

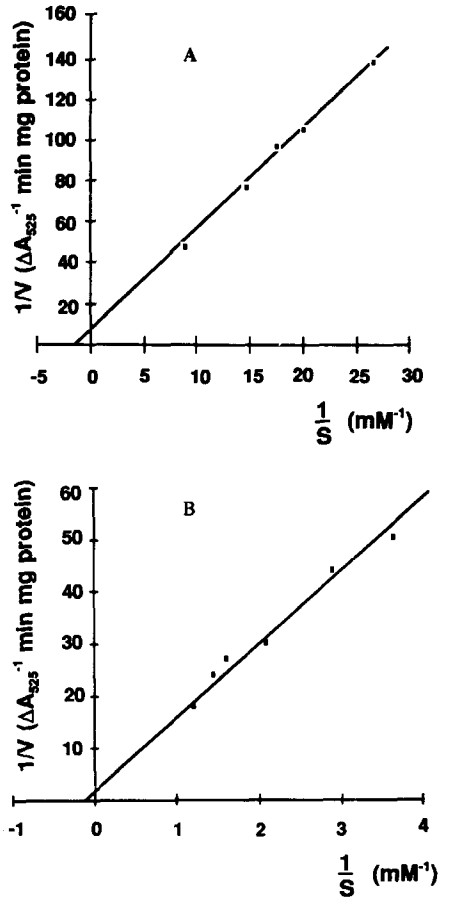

Fig. 3. Lineweaver-Burk plots of Pectinol DL activity with malvidin 3,5-diglucoside (A) and malvidin 3-monoglucoside (B) as substrates.

After 1 h of reaction, one observes a decrease in the absorbance at 330 nm, which indicates the degradation of the chalcone form.

The kinetic study with malvidin 3-monoglucoside and malvin as substrates, presented in Fig. 3, shows that Pectinol DL affinity is more important for monoglucoside (K_m = 0.72 mM) than for diglucoside (K_m = 3.2 mM). According to the works of Huang (1955) and Blom (1983), the K_m obtained with 3-monopelargonidin and 3-monocyanidin as substrates were, respectively, 0.2 mM and 0.12 mM at pH 4 and 30°C. In our experimental conditions (pH 3 and 25°C) the K_m for malvidin 3-monoglucoside was higher

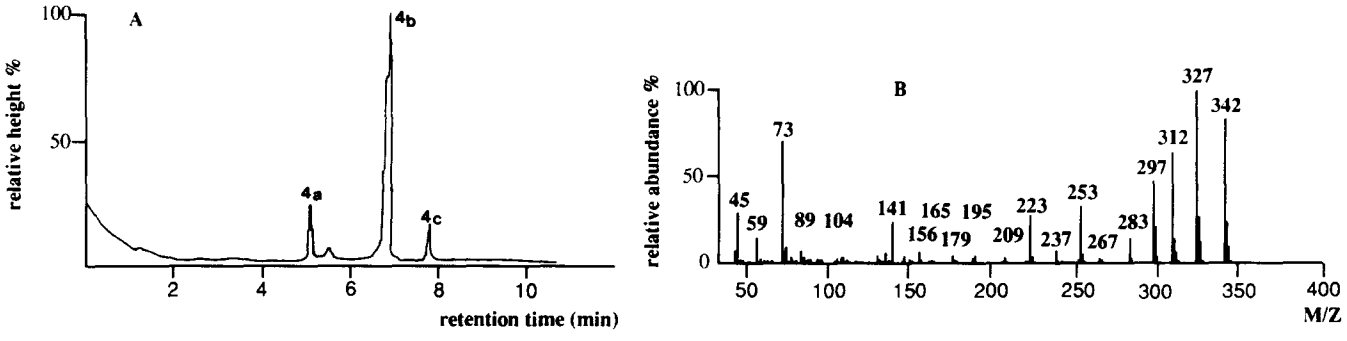

Fig. 4. GC and MS characteristics of the phenolic acids extract (PAF) resulting from malvin degradation by Pectinol DL: (A) GC chromatogram of the PAF; (B) mass spectrum of the product giving the peak 4c.

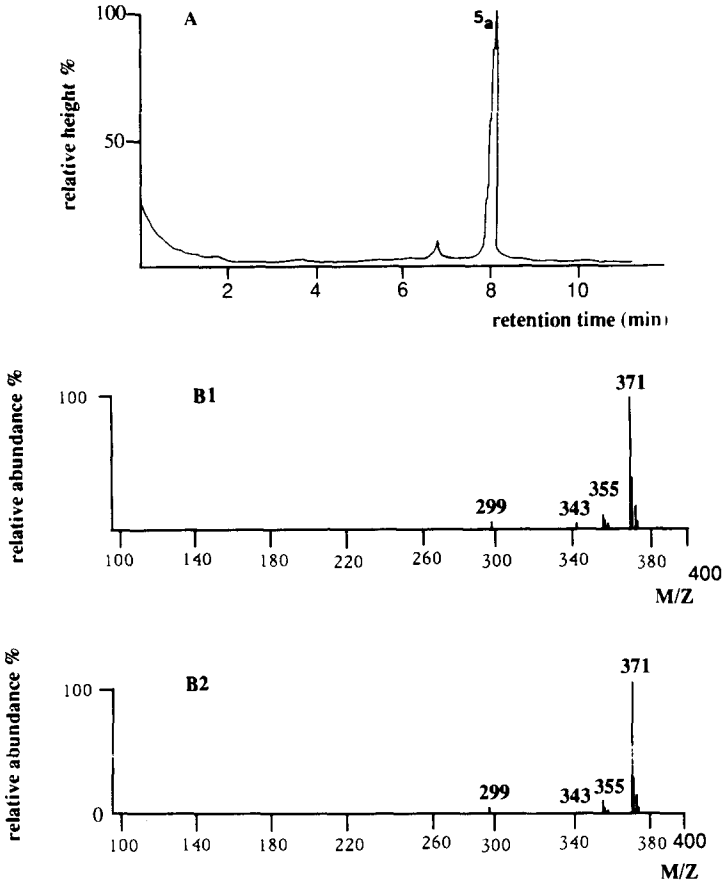

Fig. 5. GC and MS characteristics of the phenols extract (PF) resulting from malvin degradation by Pectinol DL: (A) GC chromatogram of the PF; (B) mass spectra of the product giving the peak 5a (B1) and of standard 2,4,6-trihydroxybenzaldehyde (B2).

(0.72 mM). This result is not directly comparable with those presented above since our experimental conditions were less favourable to enzyme activity. However, it is anticipated that this K_m (0.72 mM) would be higher than the K_m obtained with the monoglucosides of pelargonidin and cyanidin. It seems, therefore, that enzyme affinity is increased by the hydrophilic character of the aglycone.

The enzymatic breakdown (Pectinol DL) of glycosidic bonds finally leads to rapid destruction of the corresponding aglycone. However, when a purified aglycone (malvidin) is subjected to the same conditions (pH 3 and 25°C), the degradation takes place very slowly.

After complete decolorisation of the reaction medium during 12 h storage in the dark, the breakdown products were analysed by chromatographic and spectrometric methods.

From the PAF fraction, syringic acid was identified by comparing R_F values (TLC) and retention time (GC) with standard values. It can be observed by GC analysis of the PAF fraction, that there are three significant peaks (Fig. 4(A)). Peak 4b was identified by EI mass spectrometry as citric acid (m/z = 465). Citric acid was one of the constituents of the buffer used for enzymatic reaction. Peak 4c was identified from its mass spectrum as syringic acid as represented in Fig.4(B). Peak 4a was not identified.

From the fraction containing phenols (PF) peak 5a (Fig. 5(A)) was identified as 2,4,6-trihydroxybenzaldehyde with an m/z value of 371 by CI mass spectrometry (Fig. 5B). Aldehydic character was clearly confirmed by positive reaction with DNPH on TLC (Mehlitz *et al.*, 1963).

A possible scheme of malvin breakdown, by Pectinol DL in aqueous solution at pH 3, is presented in Fig. 9 (see below). The last products of malvin degradation were identified as syringic acid and 2,4,6-trihydroxybenzaldehyde. The increase in absorbance at 330 nm in the first minutes after addition of enzyme is indicative

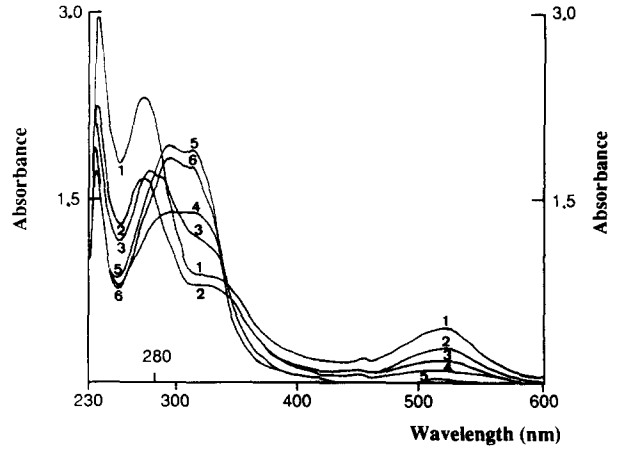

Fig. 6. Absorbance spectra of malvidin 3,5-diglucoside during its thermal degradation. Scans 1–6: malvin measured after 0.25, 1, 3, 6, 12 and 15 h of reaction.

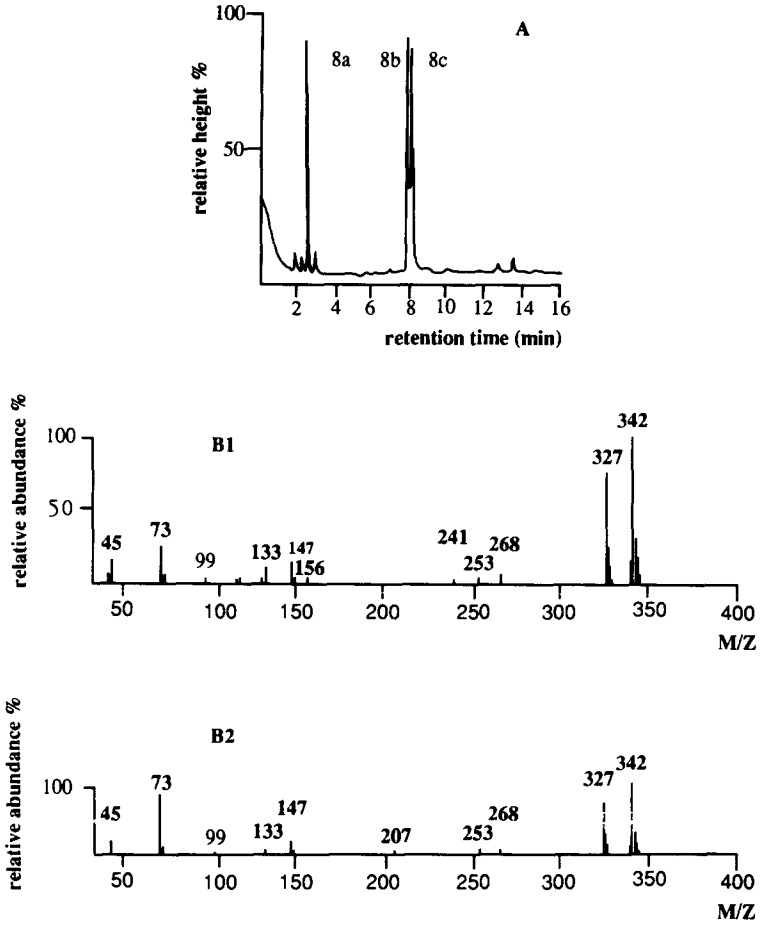

Fig. 7. GC and MS characteristics of the phenols extract (PF) resulting from thermal degradation of malvin: (A) GC chromatogram of the PF; (B) mass spectra of the product giving the peak 8a (B1) and of standard phloroglucinol (B2).

of chalcone formation as an intermediate of degradation (Preston & Timberlake, 1981). However, malvidin submitted to enzymatic action under the same conditions is only degraded very slowly. This observation suggests that enzymatic hydrolysis of glycosidic bonds leads to a very unstable form of aglycone, named 'X', which breaks off very quickly in two parts (syringic acid and 2,4,6-trihydroxybenzaldehyde) with loss of an atom of carbon. This loss, possibly occurring after hydroxylation or oxidation of chalcone, remains unexplained.

The thermal degradation of malvin was also examined. Figure 6 shows the absorbance spectra of malvin solution (pH 3) recorded after increased times of heating at 100°C. It can be seen that the anthocyanin equilibrium is shifted from the flavylium form to the chalcone form when the heating time is increased (Preston & Timberlake, 1981). After extraction from the reaction medium, fractions containing phenolic acids (PAF) and neutral phenolic compounds (PF) were analysed by gas chromatography. Certain peaks (7a and 8b) gave the same mass spectrum as peak 4c (previously identified as syringic acid). The compound corresponding to peak 8a (Fig.7(A)) displayed the same mass spectrum (Fig.7(B)) as the phloroglucinol standard. The product represented by peak 8c was identified according to its mass spectrum (EI) as 2,4,6-trihydroxybenzaldehyde (Fig.8).

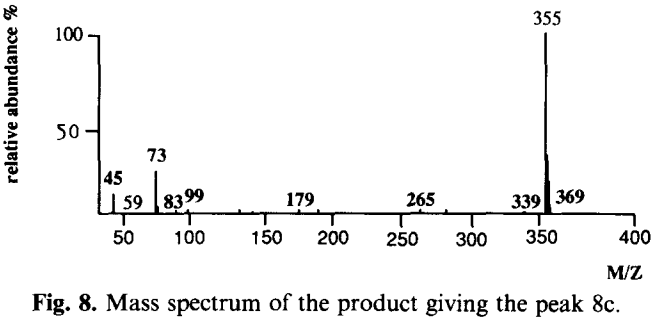

Fig. 8. Mass spectrum of the product giving the peak 8c.

The ultimate breakdown products of malvin, identified after thermal treatment, were the same as found after enzymatic degradation. The results can be summarised by the hypothetical pathway presented in Fig.9. The formation of phloroglucinol under more drastic conditions (100°C for 15 h) can be explained by oxidation of 2,4,6-trihydroxybenzaldehyde to 2,4,6-trihydroxybenzoic acid and then decarboxylation.

In conclusion, enzymatic degradation of malvin starts by hydrolysis of glucosidic bonds which destabilises the aglycone and leads to the ring opening. It can be seen then, that the degradation pathways are the same for both enzymatic and thermal treatments. Maccarone *et al.* (1987) made similar observations after photodegradation of several anthocyanins, ascrib-

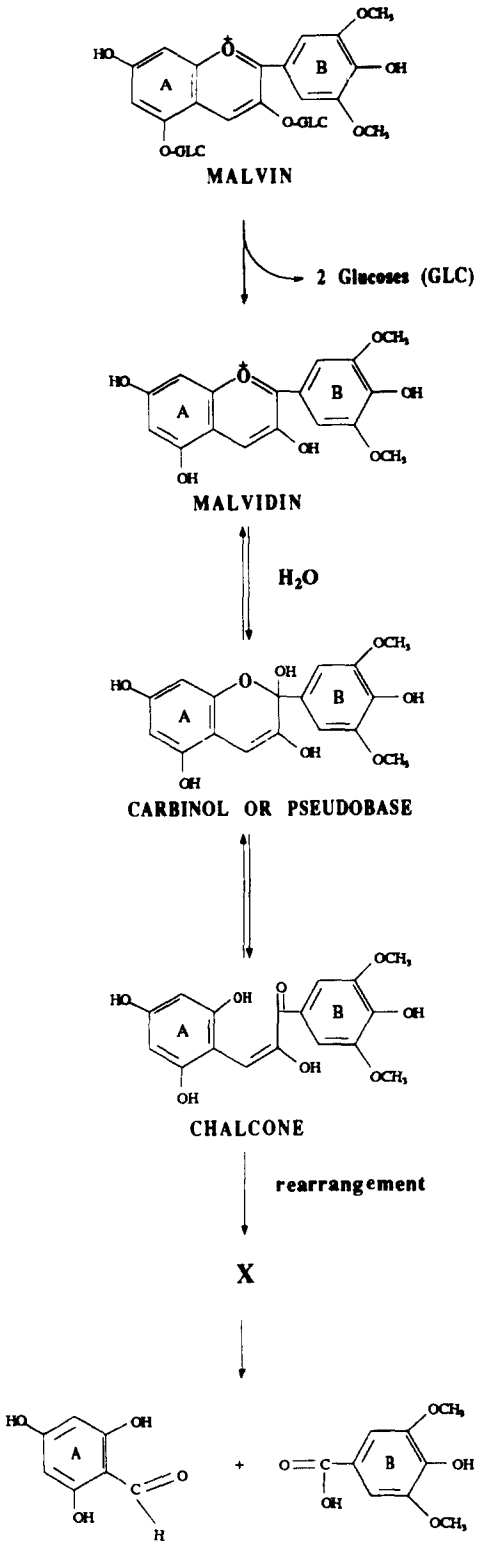

Fig. 9. Hypothetical scheme of malvin degradation pathway by Pectinol DL and thermal treatments.

ing these results to ring opening of 2,4,6-trihydroxychalcone and formation of carboxylic acids and carboxyaldehydes by proton transfer and rearrangements in the acidic aqueous medium. Such a free radical mechanism could explain the end products formed by thermal treatment. This explanation is less valid for enzymatic breakdown of malvin.

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