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TYROSINE AND CYSTEINE ARE SUBSTRATES FOR BLACKSPOT SYNTHESIS IN POTATO

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Key Word Index—*Solanum tuberosum*; Solanaceae; potato; blackspot; bruise; melanin; polyphenol oxidase; enzymic browning

Abstract—Partially purified blackspot pigments from potato tubers (*Solanum tuberosum* L.) of two commercial cultivars were subjected to a microassay for melanin, which consisted of specific chemical degradation and subsequent HPLC analysis. Permanganate oxidation yielded pyrrole-2,3,5-tricarboxylic acid, whereas hydrolysis in hydriodic acid liberated aminohydroxyphenylalanine isomers. These results indicate that the polymeric pigments, which have previously been found to contain a protein matrix, carry crosslinked 5,6-dihydroxyindole-2-carboxylic acid and benzothiazine units. This leads to the conclusion that free tyrosine and free cysteine are incorporated in the proteinaceous pigments via the polyphenol oxidase catalysed pathway of melanogenesis in the process of blackspot formation. The findings are in accordance with the hypothesis that the process of blackspot formation is a non-regulated cascade of reactions in disintegrated tuber cells, rather than a finely tuned biosynthesis.

INTRODUCTION

Application of mechanical impacts to potato tubers (*Solanum tuberosum* L.), e.g. during harvest or transport, may induce the development of dark patches beneath the intact tuber skin. This quality problem, generally referred to as blackspot, yearly causes substantial economic damage to potato breeders and the potato industry. The key enzyme in the synthesis of blackspot pigments is polyphenol oxidase (PPO), as has been proven by means of antisense PPO gene expression [1].

Since L-tyrosine is the main PPO substrate in potato tubers [2], blackspot pigments have generally been considered to be tyrosine-derived melanin polymers [3]. This assumption is supported by the observation that tyrosine is the main determinant for the potential of tuber tissue to synthesise blackspot pigments [4–7]. The PPO catalysed oxidation of tyrosine leads to the formation of dopaquinone. In the biosynthesis of mammalian melanins this intermediate may be polymerised via two major pathways (Scheme 1). Intramolecular nucleophilic addition of the amino group gives the indole derivative leucodopachrome which after polymerisation ultimately leads to the black eumelanin. In the presence of thiol compounds thioether derivatives of dopa are formed; the reaction with cysteine yields cysteinyldopa, which after further oxidation and polymerisation finally gives the reddish-brown phaeomelanin. Consequently, eumelanin is mainly composed of 5,6-dihydroxyindole and 5,6-dihydroxyindole-2-carboxylic acid (DHICA) units, whereas phaeomelanin mainly contains benzothiazine units (Scheme 1) [8–11].

A characterisation study of blackspot pigments isolated from two commercial cultivars showed that the isolated pigments contained a protein matrix with covalently bound constituents which give the polymers an absorbance throughout the visible spectrum. The results indicated that the pigments of one of the cultivars incorporated the endogenous *o*-diphenol chlorogenic acid [12]. However, attempts to demonstrate tyrosine incorporation indirectly by means of a spectrophotometric assay for eumelanin were unsuccessful [12]. In order to obtain conclusive experimental evidence for the generally accepted theory that tyrosine is a substrate for blackspot formation, we attempted to detect DHICA and benzothiazine units in blackspot pigments. We therefore subjected the pre-

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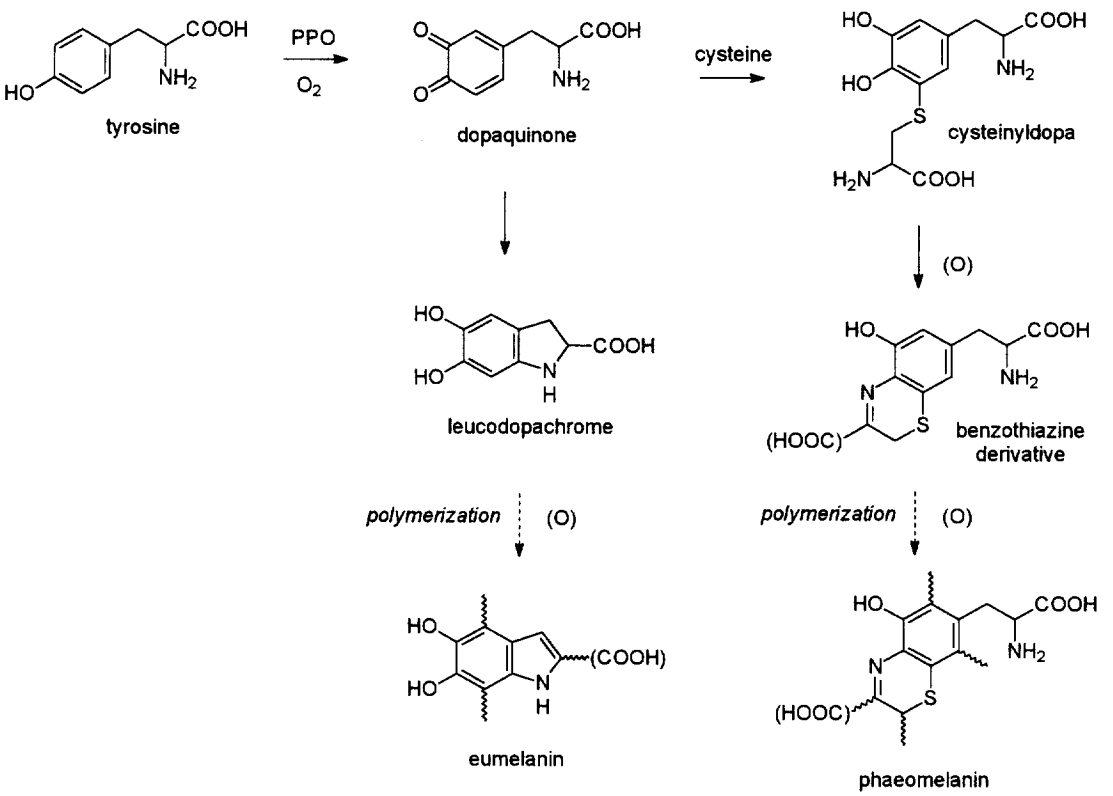


Scheme 1. PPO catalysed oxidation of tyrosine and subsequent conversion reactions and polymerisation leading to eumelanin and phaeomelanin, which are mainly composed of dihydroxyindole and benzothiazine subunits, respectively [10].

viously described samples [12] to a microassay which was originally developed for the analysis of melanin in animal tissue [9]. The assay is based on the formation of pyrrole-2,3,5-tricarboxylic acid (PTCA) by permanganate oxidation of DHICA containing pigments, and on the formation of aminohydroxyphenylalanine (AHP) isomers by hydrolysis of benzothiazine containing pigments in hydriodic acid (Scheme 2). Generally, these procedures yield *ca* 2% of the theoretical PTCA and *ca* 20% of the theoretical AHP, respectively, as calculated from the amount of DHICA and benzothiazine subunits present in the melanin polymers [9, 13]. PTCA and AHP isomers were quantified by HPLC.

RESULTS AND DISCUSSION

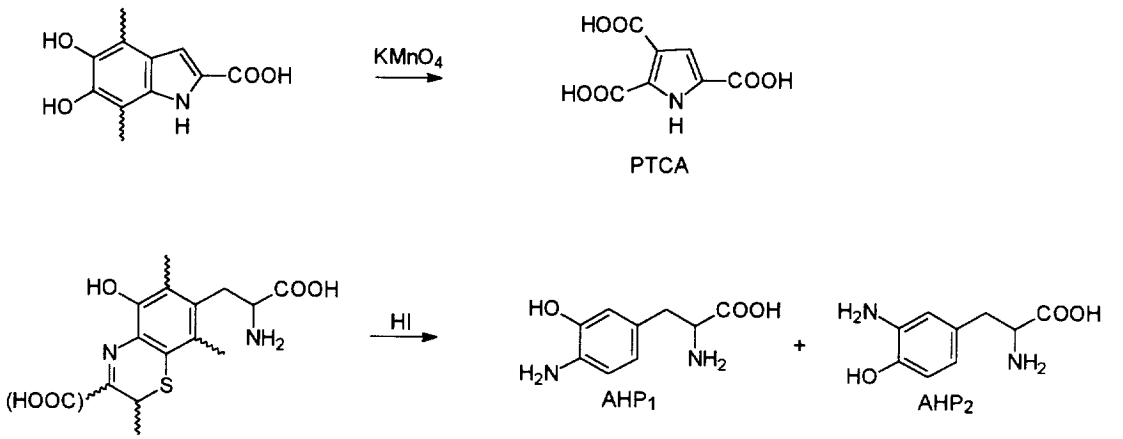
Determination of PTCA

Partially purified samples of undamaged (control) and genuine blackspot tuber tissue of potato cultivars Lady Rosetta and Bildtstar were degraded by permanganate oxidation. The degradation products were analysed for the presence of PTCA using HPLC with fluorimetric detection (HPLC system I). The chromatograms of the blackspot samples of both cultivars showed a significant peak with the retention time of PTCA (8.25 min; Fig. 1a). In contrast, the chromatograms of the samples derived from the undamaged control tissue only showed a very small peak at 8.25 min(Fig. 1b). Addition of PTCA to the degradation products resulted in a corresponding increase of this HPLC peak. The identity of the degradation product was confirmed using a second HPLC system with different column, eluent and detection system (HPLC system II). Table 1 shows the amount of PTCA in the four samples as quantified by HPLC system I.

Since the PTCA yield by permanganate oxidation of DHICA-derived eumelanin is *ca* 2% [13], a rough estimate of the dopaquinone-derived indole monomer content may therefore be made by multiplying the PTCA values by a factor 50. This leads to *ca* 7.5 nmol of crosslinked indole monomers per mg of partially purified pigments of Lady Rosetta, and to *ca* 6.5 nmol of crosslinked indole monomers per mg of partially purified pigments of Bildtstar.

Determination of AHP isomers

In order to determine whether the partially purified tuber samples contained benzothiazine units they were hydrolysed in hydriodic acid and subsequently analysed by HPLC with electrochemical detection (HPLC system III). The hydrolysed samples of blackspot tuber tissue isolated from both cultivars contained



Scheme 2. Formation of pyrrole-2,3,5-tricarboxylic acid (PTCA) and aminohydroxyphenylalanine (AHP) isomers by permanganate oxidation of eumelanin which contains 5,6-dihydroxyindole-2-carboxylic acid (DHICA) subunits and by hydriodic acid hydrolysis of phaeomelanin which contains benzothiazine subunits, respectively [9].

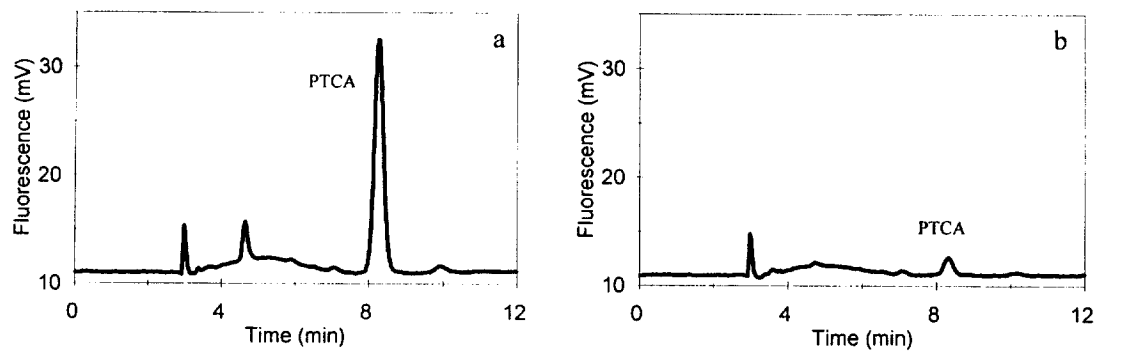


Fig. 1. HPLC analysis (HPLC system I) of blackspot (a) and control (b) samples after degradation by permanganate oxidation, showing the presence of pyrrole-2,3,5-tricarboxylic acid (PTCA). The samples were derived from tuber tissue of cultivar Lady Rosetta.

Table 1. Amount of AHP isomers and PTCA detected in chemically degraded blackspot pigments and control material from potato tubers of cultivar Lady Rosetta and cultivar Bildtstar, expressed in pmol per mg dry wt (± range in duplicate measurements) of partially purified tuber material. AHP_1: 4-amino-3-hydroxyphenylalanine; AHP_2: 3-amino-4-hydroxyphenylalanine; PTCA: pyrrole-2,3,5-tricarboxylic acid

	Lady Rosetta		Bildtstar	
	Blackspot	Control	Blackspot	Control
PTCA	156±11	9.0±0.5	133±3	4.6±0.0
AHP_1	537±47	83±2	627±6	0.0±0.0
AHP_2	280±5	106±2	96±1	21.4±0.1
AHP_{total}	817±52	188±4	722±7	21.4±0.1

considerable amounts of 3-amino-4-hydroxyphenylalanine (AHP_2) and 4-amino-3-hydroxyphenylalanine (AHP_1) with retention times of 6.65 min and 7.87 min, respectively. Fig. 2a shows the chromatogram of the blackspot hydrolysate of Lady Rosetta. The hydrolysed control sample of undamaged tissue from Lady Rosetta contained minor amounts of both AHP isomers (Fig. 2b); the control of Bildtstar contained only a small amount of AHP_2, and no detectable AHP_1 (not shown). The quantities of AHP_1 and AHP_2 present in the hydrolysates of the four samples are summarised in Table 1.

The yield of AHP isomers after the hydrolysis of phaeomelanin in hydriodic acid has been reported to be *ca* 20% [13]. The estimated amount of crosslinked benzothiazine monomers is therefore *ca* 4 nmol per mg of partially purified pigment of Lady Rosetta, and *ca* 3.6 nmol per mg of partially purified pigment of Bildtstar.

Characterisation of the chemical structure of blackspot pigments

The results indicate that blackspot pigments contain crosslinked DHICA and benzothiazine that are

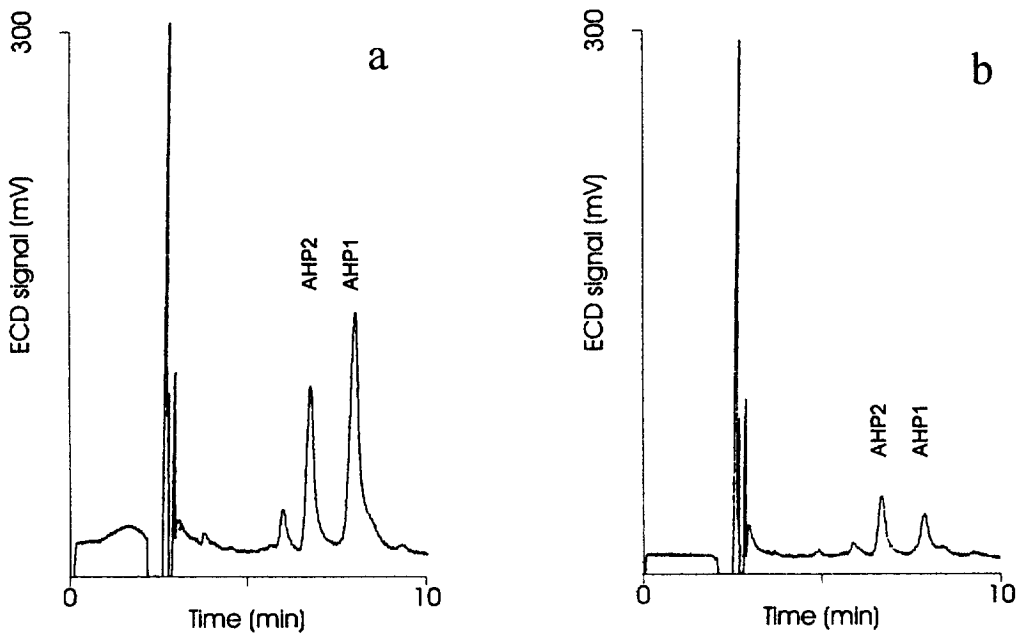


Fig. 2. HPLC analysis (HPLC system III) of blackspot (a) and control (b) samples after hydrolysis with hydriodic acid, showing the presence of aminohydroxyphenylalanine (AHP) isomers. The samples were derived from tuber tissue of cultivar Lady Rosetta.

typical subunits of mammalian eumelanin and phaeomelanin, respectively. Previously it was found that chemical hydrolysis of these blackspot pigments in hydriodic acid did not yield any detectable fragments that could be collected as insoluble precipitate. This indicated that no polymeric eumelanin was present [12]. In addition, it has been shown that the pigments can be partially solubilized by proteolytic cleavage [12]. We therefore conclude that only small oligomers of covalently bound DHICA and benzothiazine units are linked to the protein matrix of the pigments. Most probably the mechanical impact by which the synthesis of pigments is started leads to liberation of PPO from its subcellular compartment, i.e. liberation from vesicles inside the amyloplast [14]. As a result, PPO is able to catalyse the oxidation of tyrosine. The generated dopaquinone is cyclised and oxidised, resulting in crosslinked DHICA, and when it encounters free cysteine, crosslinked benzothiazine is formed (Scheme 1). Along with this oxidative polymerisation process the intermediates may react with functional groups presented by the relatively large quantity of cellular protein, in particular with the nucleophilic sulfhydryl group of protein-bound cysteine [8, 15, 16]. Also chlorogenic acid, which is the second main PPO substrate in potato tubers, can become incorporated into the pigments, as has previously been shown for pigments from cultivar Bildtstar [12]. According to this model blackspot pigments are products of non-regulated reactions in disintegrated tuber cells, rather than products of a controlled biosynthesis. This may also explain the relatively low DHICA and benzothiazine content. Taking into account the amount of pigment present in the blackspot tissue (several mg per g of dry wt, [12]), DHICA and benzothiazine content ranges in the ng scale per mg of fresh wt; the amount of DHICA and benzothiazine units in hair and melanosomes range in the μg scale per mg of fresh wt [9]. In conclusion, the results of this study provide strong experimental support for the hypothesis that in the process of blackspot formation non-protein bound tyrosine is incorporated in the polymeric pigments via the PPO catalysed pathway of melanogenesis. Furthermore, they show that also non-protein bound cysteine is involved in pigment synthesis.

EXPERIMENTAL

Blackspot pigments

Pigmented and control material have been described previously and were then referred to as "fractions IX" [12]. They were partially purified from respectively bruised and unbruised tuber tissue of two commercial potato varieties, Bildtstar and Lady Rosetta [12].

Chemicals

AHP_2 standard was purchased from Sigma. AHP_1 standard was kindly provided by Prof. S. Ito, Toyoake, Aichi, Japan. PTCA standards were obtained from Prof. Ito, Japan and Dr. J. Borovansky, Prague, Czech Republic.

Sample preparation

For the PTCA measurements 100 μl of suspended sample (20 mg dry wt per ml of H_2O) was subjected to $KMnO_4$ oxidation as previously described [9]. The Et_2O extract of the degraded material was dried, dissolved in 100 μl elution buffer and analysed by HPLC. Sample preparation for the AHP analysis was performed by a modification of the method of ref [9]. The hydrolysate obtained after overnight hydrolysis in HI at 130° was dried under a stream of N_2, redissolved in 0.05 M Li_3PO_4sol (pH 4.0) and pipetted on an aromatic sulfonic acid column (containing 100 mg of sulfonic acid silane bonded to Silicagel, 40 μm, 60 Å, Baker). After washing with one column vol of H_2O, AHP isomers were eluted with 2 ml 0.3 M KCl (pH 8.5) and analysed by HPLC.

High performance liquid chromatography

HPLC system I consisted of a Gyncotek pump model 300 and a Jasco Fluorescence detector model FP-920. Samples (20 μl) were injected onto a Waters μBondapack C18 column (300 × 3.9 mm) using a Triathlon Model 900 autosampler and eluted with 2% MeOH in 0.05 M K-Pi, pH 4.25, at a flow rate of 1 ml.min^{-1}. Excitation and emission wavelengths were 280 and 335 nm, respectively. Detector signals were recorded with an Axxiom model 717 chromatography data system (Gyncotek). HPLC system II consisted of an LDC pump (constaMetric 4100) and a LDC spectroMonitor 4100 UV detector. Samples (20 μl) were injected onto an Altech Hypersil ODS C18 3U column (150 × 4.6 mm) and eluted with 10% MeOH in 0.05 M K-Pi, pH 2.5, at a flow rate of 0.8 ml.min^{-1}. *A* was recorded at 278 nm using the Baseline 810 software program (Interscience). HPLC system III consisted of a Gyncotek pump model 300C and an ESA model 5100A Coulochem electrochemical detector with an analytical cell model 5011 (InterScience). Samples (20–50 μl) were injected with a Rheodyne 9125 injector onto a stainless steel reversed phase Superco-sil LC-18-DB, 25 × 4.6 mm analytical column (Supelchem) and eluted with 1% (v/v) MeOH in 0.01 mM K-Pi buffer pH 5.7, 1 mM Na 1-octanesulfonate and 30 mg.l^{-1} Na-EDTA, at a flow rate of 0.9 ml.min^{-1}.

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