Sources of Tyrosine in Genotypes of *Solanum tuberosum* L. Differing in Capacity to Produce Melanin Pigments

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Abstract. Potato tubers (*Solanum tuberosum*) of genotypes that vary in resistance to dark pigment formation when damaged, characteristic of the physiological disorder blackspot, were assayed for free tyrosine. The tubers were also assayed for relative levels of chorismate mutase and proteinase activities, which can regulate free tyrosine levels. The susceptibility of potato tubers to blackspot was shown to be correlated to the amount of free tyrosine by third order regression ($R = 0.88$). Tyrosine was found to be a limiting factor in pigment development. Chorismate mutase activity (CMI and CMII) was not correlated to blackspot susceptibility of the genotypes studied. Proteinase activities of Atlantic, TXA 763-5, Russet Russet, Russet Burbank, and Lemhi Russet tuber protein extracts measured with synthetic substrates correlated with blackspot susceptibility. This suggests that the high free tyrosine levels associated with blackspot susceptibility may be due to high levels of proteinase activity in the tuber, rather than tyrosine synthesis.

Solanum tuberosum tubers used commercially for a variety of consumer products are subjected to bruising during harvesting and handling. If tubers are bruised by dropping them onto hard surfaces, they may develop discolored areas referred to as blackspot bruise. The blackspot bruise is an accumulation of melanin-like pigments (Van Middelem et al., 1953) formed by oxidation of phenolic substrates by polyphenyloxidase (PPO) (Matheis, 1987a). The principle substrates for PPO in potato tubers are tyrosine and chlorogenic acid (Craft, 1966; Matheis, 1987b; Patil and Zucker, 1965). Susceptibility to blackspot bruise may be influenced by cultural practices, including irrigation and mineral nutrition (Kunkel and Gardner, 1965; Mapson et al., 1963; Mulder, 1949). Susceptibility also varies between different cultivars/genotypes and is correlated with endogenous-free tyrosine concentrations in the tuber (Corsini et al., 1992; Dean et al., 1993; Mapson et al., 1963; Sapers et al., 1989; Stark et al., 1985; Tripathi et al., 1983). Blackspot bruise susceptibility does not correlate with chlorogenic acid or other phenolic components in the tuber (Corsini et al., 1992; Dean et al., 1993; Mapson et al., 1963).

The amount of tyrosine available for oxidation by PPO depends on tyrosine synthesis and its partitioning into protein (Dean et al., 1992). Tyrosine is synthesized via the shikimic acid pathway (Gilchrist and Kosuge, 1980), which is believed to be regulated by several key enzymes (Jensen, 1986). One such enzyme is chorismate mutase (CM), of which two isozymes (CMI and CMII) have been characterized (Gilchrist et al., 1972; Goers and Jensen, 1984; Kuroki and Conn, 1988; Singh et al., 1986).

The quantity of tyrosine found in the free, readily oxidizable pool may also be affected by the relative rates of protein synthesis and degradation. Since potato cultivars vary considerably in protein content and protein content has been correlated with blackspot resistance (Corsini et al., 1992; Stark et al., 1985), the levels of

proteinase activity may be important in tyrosine availability and subsequently blackspot resistance. It is important to understand how tyrosine synthesis and partitioning contribute to the soluble pool of tyrosine in potato tubers. Since our previous work indicated that the amount of protein decreased at the same time as free tyrosine and pigment formation increased (Dean et al., 1993), we decided to look at protein hydrolysis as a potential source of tyrosine. The purpose of this report is to examine the relative activities of chorismate mutase and proteinase activity in resistant and susceptible potato genotypes.

Materials and Methods

Potato tuber production in the field. Potato tubers from 14 cultivars varying in blackspot susceptibility were obtained from the U.S. Dept. of Agriculture (USDA) breeding program at Aberdeen, Idaho. The cultivars were genotypes selected from a diallel used to examine the heritability of blackspot susceptibility (Table 1) (Dean et al., 1993; Pavek et al., 1993). The tubers were stored briefly at 2C until needed for tyrosine determinations. The basal end of each tuber (≈ 10 to 20% the length of the tuber) was removed for blackspot susceptibility and tyrosine analysis.

A second group of five cultivars was selected based on their bruise susceptibility and grown for the purpose of enzyme assays. This group consisted of Russet Burbank, Lemhi Russet, Atlantic, Ranger Russet, and TXA 763-5. They were planted in early April at Washington State University, Prosser, Irrigated Agriculture Research and Extension Center (IAREC) and grown using standard commercial production practices. All the plants were harvested following maturation (vine senescence) and placed in storage at 2C briefly until the assays were conducted. The average tuber size varied by cultivar so average-sized tubers were selected from each cultivar.

Blackspot susceptibility measurement. The susceptibility of tuber tissue to blackspot development was determined by homogenizing half of the basal portion of the tuber (≈ 10 to 20% of the tuber) according to the homogenization procedure outlined previously (Dean et al., 1993). Between 20 and 50 g of tuber tissue was homogenized with 2x v/w 0.05 M phosphate buffer, pH 6.5, and allowed to oxidize for 24 h at room temperature. The oxidized

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Table 1. Cultivars from the U.S. Dept. of Agriculture breeding program at Aberdeen. OD₄₇₅ of control homogenates and endogenous-free tyrosine levels are provided for each cultivar. Each value represents the mean of three replicates $\pm$ SE.

Genotype	OD ₄₇₅	Free tyrosine ($\mu\text{g}\cdot\text{g}^{-1}$)
A84685-14	0.147 $\pm$ 0.013	71.9 $\pm$ 6.2
A84707-16	0.155 $\pm$ 0.039	79.5 $\pm$ 4.6
TXA 763-5	0.165 $\pm$ 0.031	63.0 $\pm$ 4.5
A7938-1	0.340 $\pm$ 0.045	90.0 $\pm$ 5.2
A75188-3	0.371 $\pm$ 0.070	113.9 $\pm$ 3.7
A7816-14	0.387 $\pm$ 0.046	112.0 $\pm$ 5.7
875PX15-1	0.456 $\pm$ 0.087	196.4 $\pm$ 36.3
A84727-60	0.470 $\pm$ 0.108	161.7 $\pm$ 7.0
A81473-2	0.523 $\pm$ 0.080	189.2 $\pm$ 16.0
Belrus	0.531 $\pm$ 0.046	229.5 $\pm$ 16.0
Norchip	0.690 $\pm$ 0.121	273.9 $\pm$ 38.1
A84537-42	0.710 $\pm$ 0.073	220.8 $\pm$ 11.5
Lemhi Russet	0.755 $\pm$ 0.032	172.5 $\pm$ 18.7
Russet Burbank	0.856 $\pm$ 0.109	374.0 $\pm$ 60.2
ATD 63-7	0.962 $\pm$ 0.175	236.0 $\pm$ 18.5

homogenate was filtered and centrifuged, and the optical density of the supernatant was measured at 475 nm. The second half of the basal end of the tuber was frozen at -80°C for either tyrosine analysis or protein extraction for enzyme assays. Each sample consisted of three randomly selected tubers and was replicated four times.

Tyrosine analysis. The frozen tuber tissues obtained from the U.S. Dept. of Agriculture breeding program samples were lyophilized to dryness with a Freezmobile (model 125L; Vertis Co., Gardner, N.Y.), ground with a Wiley Mill (Arthur Thomas Co., Philadelphia), and passed through a 40-mesh screen. The dry, ground samples were further powdered with a 'Wig-L-Bug' powderer (Crescent Dental Manufacturing Co., Chicago) for extraction. The samples were extracted and analyzed by high-performance liquid chromatography (HPLC) using the 'Pico Tag' procedure as previously reported (Dean et al., 1993).

Substrate additions. A subsample of six genotypes with varying amounts of free tyrosine was selected to measure the effect of exogenously added tyrosine on total oxidation of homogenized potato tuber tissue. Samples were assayed to determine endogenous oxidative enzyme (PPO) substrate. These measurements were also used to determine if PPO activity was limiting blackspot pigment formation. The subsample was selected from the U.S. Dept. of Agriculture breeding program samples (Lemhi Russet, TXA 763-5, A75188, 875PX15, A84531, and A84727). Between 50 and 75 g of basal end tuber tissue from each genotype was homogenized with 2x v/w 0.05 M phosphate buffer, pH 6.5. The homogenate was then filtered through filter paper (Whatman No. 1; Hillsboro, Ore.) and centrifuged at 13,220 g for 10 min. Tyrosine was added to the supernatants at 0 or 400 μg per gram fresh weight of potato tissue. The homogenates were then allowed to oxidize overnight at room temperature. The increase in pigment formation over the control (without added tyrosine) was measured spectrophotometrically at 475 nm.

Protein extraction for enzyme assays. Half of the basal portions of two to four tubers from each 1992 field harvest genotype was removed, placed in a plastic zip-lock bag, and frozen immediately at -80°C . Four replicate samples from each treatment were collected. The frozen sample was diced and 5.0 g of the sample was homogenized in a cold mortar with 20% insoluble polyvinyl-pyrrolidone (PVP) in 30 ml cold 0.1 M phosphate buffer, pH 6.5, containing 5 mM dithioerythritol (DTE). The homogenized tissue

was then transferred to a 40-ml glass tissue grinder with 10 ml cold buffer for final homogenization. The homogenate was centrifuged at 29,000 g for 20 min at 6°C . Proteins in the supernatant were precipitated with ammonium sulfate at 85% saturation with stirring for 20 min. The samples were centrifuged for 20 min at 29,000 g to obtain the protein pellet. The final pellet was resuspended in 5 ml cold 0.05 M phosphate buffer, pH 6.5 containing 1 mM DTE. The samples were then desalted by passing them through a column (model Sephadex G-25; Pharmacia Inc., Alameda, Calif.) with the same buffer. All procedures were carried out at 6°C . The final extract represented 0.71 $\text{g}\cdot\text{ml}^{-1}$ and all results are presented on a fresh-tissue-weight basis. This protein extract was used in the proteinase and chorismate mutase assays.

Chorismate mutase assay. The assay mixture for total chorismate mutase activity (CMI + CMII) contained 200 μl protein extract, 100 μl 0.1 M phosphate buffer, pH 7.0, 100 μl 2 mM chorismic acid (dissolved in 0.1 M phosphate buffer, pH 7.0) and 100 μl 1 mM L-tryptophan. The final concentrations were 400 μM for chorismic acid and 200 μM for L-tryptophan. Activity of the unregulated isozyme CMII was measured by substituting 100 μl buffer for tryptophan. The difference between these two activities was taken as the CMI activity (Singh et al., 1986). The reaction mixture was incubated for 20 min at 25°C after which it was terminated by addition of 100 μl 6 N HCl. The samples were incubated at room temperature for 10 min to convert prephenate to phenyl-pyruvate followed by addition of 400 μl 4 N NaOH. The absorption of phenyl-pyruvate was measured at 320 nm with 1 N NaOH as a reference (Kuroki and Conn, 1988; Singh et al., 1986). All results were expressed on a fresh-weight basis so that approximate in vivo activities could be compared between genotypes with widely different protein and dry-matter concentration.

N-Benzoyl-L-arginine p-nitroanilide (L-BAPNA) endopeptidase assay. A 200- μl aliquot of each sample protein extract was added to 250 μl of 0.1 M phosphate buffer, pH 7.0, and 50 μl 2 mM L-BAPNA [dissolved in 20% dimethylformamide (DMF)]. Control samples containing either no substrate or no extract were also assayed. The final concentration of L-BAPNA was 200 μM . The samples were incubated for 40 min at 30°C after which the reactions were terminated with 500 μl 30% acetic acid. Absorbance was measured at 405 nm according to Santarius and Belitz, 1978. The activity is expressed as nmoles p-nitroaniline $\text{g}^{-1}\cdot\text{min}^{-1}$.

L-Phenylalanine-2-naphthylamide (L-Phe-NA) aminopeptidase assay. Aminopeptidase activity utilizing the L-Phe-NA substrate was determined by adding 100 μl protein extract to 500 μl 0.1 M phosphate buffer, pH 7.0, and 200 μl 1 mM L-Phe-NA (dissolved in 20% DMF). The final concentration of L-Phe-NA was 250 μM . The mixture was incubated for 30 min at 30°C , after which it was terminated by adding 200 μl 50% trichloroacetic acid (TCA). The protein was pelleted by centrifugation for 10 min at 16,000 g after precipitation for 20 min at 4°C (Waters and Dalling, 1979). The enzymatic product was measured after diazotization by incubating the supernatant with 1 ml 0.1% NaNO_2 for 3 min followed by incubation with 0.5% ammonium sulfamate for 2 min, after which 2 ml 0.05% N-naphthylethylenediamine (in 95% ethanol) was added. The absorption by the azo-dye reaction product was measured at 540 nm after 15 min (Blackwood and Mandl, 1961). The results were expressed as nmoles 2-naphthylamine $\text{g}^{-1}\cdot\text{min}^{-1}$.

L-Lysine-2-naphthylamide (L-Lys-NA) aminopeptidase assay. The reaction mixture consisted of 150 μl protein extract, 550 μl 0.1M Tris-HCl buffer (pH 8.0) and 100 μl 2 mM L-Lys-NA (dissolved in hot H_2O). The final concentration of L-Lys-NA was 250 μM . The assay conditions and determination of activity are the same as for the L-Phe-NA reported above.

Fig. 1. The percent increase in optical density (OD_{475}) of crude tissue homogenates with additions of $400 \mu\text{g}$ tyrosine/g tissue compared to the level of endogenous free tyrosine. The values plotted are means of three replications $\pm$ SE. Cultivars used are listed in Methods.

Results

Pigment development in tuber tissues. The amount of tyrosine in the soluble fraction of extracts from potato tubers from the U.S. Dept. of Agriculture breeding program correlated positively and significantly by third order regression ($90.06 - 356.59x + 2000x^2 - 1000x^3$) with the susceptibility of homogenized tuber tissue to pigment development measured by optical density at 475 nm ($r = 0.88$). Table 1 lists the cultivars used in this experiment, with their OD_{475} and free tyrosine levels. The percent increase in OD at 475 nm when tyrosine was added to homogenates from the six cultivars selected from the USDA breeding program at $400 \mu\text{g}\cdot\text{g}^{-1}$ equivalents was largest with cultivars that were lowest in endogenous-free tyrosine (Fig. 1). The percent increase in OD_{475} decreased with increasing endogenous free tyrosine until it reached a saturation point between 162 and $225 \mu\text{g}\cdot\text{g}^{-1}$ tyrosine. The percent increase at this saturation level was $\approx 30\%$ with $400 \mu\text{g}\cdot\text{g}^{-1}$ added tyrosine.

Chorismate mutase (CM) activity. The chorismate mutase activities of protein extracts from the five cultivars grown at the IAREC are shown in Table 2. There was no significant difference in CMI, CMII, or total CM activity measured from extracts of susceptible cultivars compared to resistant cultivars. There was no significant correlation between blackspot susceptibility (as deter-

Fig. 2. The rate of endopeptidase activity from blackspot susceptible and resistant cultivars measured with L-BAPNA as the substrate compared to the OD_{475} of homogenate solutions from the following blackspot susceptible and resistant potato cultivars: Atlantic (●), TXA 763-5 (○), Ranger Russet (▽), Lemhi Russet (■), and Russet Burbank (□). The values plotted are the means of four replications $\pm$ SE.

mined by the OD_{475} of the homogenate) and CM activity ($r = 0.13$).

Proteinase activity. The endopeptidase activity measured by use of L-BAPNA as substrate correlated positively with the blackspot susceptibility of the genotype ($r = 0.78$) (Fig. 2). The endopeptidase activity determined from Atlantic and TXA 763-5 tuber tissue extracts was 45% of the activity from Lemhi Russet tubers, but not significantly different from Russet Burbank or Ranger Russet. The aminopeptidase activity was measured with two substrates, L-Phe-NA and L-Lys-NA (Fig. 3). The enzyme activity measured with L-Phe-NA as substrate was 400% greater than the rate with L-Lys-NA and about 1400% greater than the endopeptidase activity with L-BAPNA. The aminopeptidase activity measured with L-Phe-NA correlated positively with the blackspot susceptibility of the cultivars ($r = 0.92$). The aminopeptidase activity utilizing L-Lys-NA as the substrate did not correlate positively with blackspot susceptibility.

Discussion

The propensity of potato tuber tissue to develop dark-colored melanin-like pigments was highly correlated to the amount of endogenous-free tyrosine in the tissue. The total amount of pigment produced seems to be related to the concentration of soluble

Table 2. Chorismate mutase activity for protein extracts derived from five different cultivars. Values represent the means of four replicates $\pm$ SE, and are compared to the mean $OD_{475} \pm$ SE of the corresponding homogenate. Those values with the same superscript letter in a column are not different by independent *t* test (LSD) at the 0.05 level. $P < 0.01$.

Cultivar	OD_{475}	CMI	CMII	Total CM
Atlantic	0.415 ± 0.42^a	5.05 ± 0.65^{ab}	5.55 ± 0.56^b	10.60 ± 0.35^b
TXA 763-5	0.475 ± 0.023^a	4.32 ± 0.40^a	4.41 ± 0.47^b	8.73 ± 0.82^b
Ranger Russet	1.114 ± 0.116^b	7.85 ± 0.52^c	8.72 ± 1.51^c	16.56 ± 1.30^c
Lemhi Russet	1.494 ± 0.243^c	6.62 ± 0.60^{bc}	3.79 ± 0.24^{ab}	10.41 ± 0.41^b
Russet Burbank	1.599 ± 0.182^c	4.24 ± 0.74^a	1.91 ± 0.40^a	6.15 ± 0.85^a

Fig. 3. The rate of aminopeptidase activity from blackspot resistant and susceptible cultivars using L-Phe-NA (—) and L-Lys-NA (----) as substrate compared to the OD (475) of homogenate solutions from blackspot susceptible and resistant potato cultivars. The values plotted are the means of four replications SE. The following cultivars are represented: Atlantic (●), TXA 763-5 (○), Ranger Russet (▽), Lemhi Russet (□), and Russet Burbank (■).

tyrosine in a quadratic fashion (Table 1). Corsini et al. (1992) showed a linear correlation between endogenous free tyrosine and blackspot susceptibility measured by an abrasive peel method.

Polyphenyloxidase is believed to be the enzyme that catalyzes the conversion of phenolic compounds into the melanin-like pigments responsible for blackspot (Matheis, 1987a). Oxidase activity (presumably PPO) was not a limiting factor in the production of melanin-like pigments from tyrosine in this study (Fig. 1). Addition of 400 µg tyrosine/g tissue resulted in an increase of at least 31% for the OD₄₇₅ of tuber homogenates, even for the cultivars most susceptible to blackspot (Lemhi Russet and A84531). For the most resistant cultivar, TXA 763-5, the OD₄₇₅ increased by 180% when tyrosine was added. The increase in OD₄₇₅ was most pronounced for cultivars with low endogenous-free tyrosine levels. This demonstrates that the substrates such as tyrosine are the primary limiting factor in pigment production, not PPO activity. This is in agreement with results reported by Weaver et al. (1970) that there was no relationship between PPO activity and blackspot in Russet Burbank tuber tissue.

The in vitro amount of activity of one of the key enzymes in tyrosine synthesis, chorismate mutase, does not explain why the free tyrosine concentration is higher in the susceptible cultivars than the resistant cultivars (Table 2). Therefore, our previous finding that incorporation of ¹⁴C-labeled shikimic acid into free tyrosine is greater in Lemhi Russet than TXA 763-5 (Dean et al., 1992) cannot be explained by differences in chorismate mutase activity between the cultivars.

The activity of proteinases in the blackspot resistant cultivars (TXA 763-5 and Atlantic) is lower than the susceptible cultivars (Ranger Russet, Lemhi Russet, and Russet Burbank) with the endopeptidase substrate (Fig. 2) and one of the two aminopeptidase substrates tested (L-Phe-NA, Fig. 3). This is significant, since aminopeptidase activity with L-Phe-NA was greater than any

other substrate tested. The relative activity of these proteinases may at least in part explain why the protein-bound tyrosine content in TXA 763-5 is usually 1.5 to 2 times higher than that of Lemhi Russet and Russet Burbank (Corsini et al., 1992).

Previous results showed that ¹⁴C-Shikimic acid incorporation into free tyrosine is 50% greater in Lemhi Russet tuber discs compared to TXA 763-5 (Dean et al., 1992). Table 2 shows that chorismate mutase activity is not responsible for the greater free tyrosine production in Lemhi Russet, nor for the greater concentrations of free tyrosine prevalent in susceptible cultivars.

The endopeptidase activity (measured with the substrate L-BAPNA) in the blackspot resistant cultivars was lower than in the susceptible cultivars, especially Lemhi Russet. The aminopeptidase activities of the susceptible cultivars measured with L-Phe-NA was ≈70% greater than the resistant cultivars. Therefore, the relative amounts of tyrosine found in the soluble pool from potato tuber tissue that can be used as a substrate by PPO. The amount of potential protein hydrolysis by endogenous proteinase correlates well with the blackspot susceptibility (melanin-like pigment formation) of cultivars used in this study.

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