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, characterized 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, Ranger 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.

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 cross 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 2°C 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 2 x v/w 0.05 M phosphate buffer, pH 6.5, and allowed to oxidize for 24 h at room temperature. The oxidized 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.

'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 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).

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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 the supernatant with 1 ml 0.1% NaNO2 for 3 min followed by precipitation for 20 min at 4°C (Waters and Dalling, 1979). The proteins in the supernatant were precipitated with ammonium sulfate at 85% saturation with stirring for 20 min. 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 g·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% (v/v) 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 Bellitz, 1978. The activity is expressed as nmoles p-nitroaniline g⁻¹ 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% NaNO₂ for 3 min followed by incubation with 0.5% ammonium sulfamate for 2 min, after which 2 ml 0.05% N-(naphthyl)ethylenediamine (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 g⁻¹ min⁻¹.

L-Lysine-2-naphthylamide (L-Lys-NA) aminopeptidase assay. The reaction mixture consisted of 150 µl protein extract, 550 µl 0.1x Tris-HCl buffer (pH 8.0) and 100 µl 2 mM L-Lys-NA (dissolved in hot H₂O). 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.
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 proteins in the tissue. The total amount of pigment production is inversely proportional to the amount of free tyrosine in the tissue. This suggests that the amount of free tyrosine in the tissue is a key factor in the development of dark-colored melanin-like pigments.

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 µg·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 µg·g$^{-1}$ tyrosine. The percent increase at this saturation level was ≈30% with 400 µg·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 determined by the OD$_{475}$ of the homogenate) and CM activity ($r = 0.13$).

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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.

Polyphenol oxidase 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 least 31% for the OD475 of tuber homogenates, even for the cultivars most susceptible to blackspot (Lemhi Russet and A84531).

The activity of proteinases in the blackspot resistant cultivars was lower than in the susceptible cultivars, especially Lemhi Russet. The aminopeptidase activity of the susceptible cultivars measured with L-Phe-NA was about 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.

**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 (■).

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