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Comparison of Accumulation of Capsaicinoid Contents with Capsaicinoid Synthetase Activity at Different Developmental Stages of *Capsicum annuum* L.

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The contents of various capsaicinoids viz. nordihydrocapsaicin (NDC), capsaicin (CAP), and dihydrocapsaicin (DHC) were determined in different parts of fruits (placenta, pericarps, seeds, and whole fruits) at different developmental stages after flowering and compared with the capsaicinoid synthetase (CS) activity. The capsaicinoid contents were very low up to 24 days after flowering (DAF), and there was a significant increase at 36 DAF in all parts of fruits. The enzyme activity of placenta increased to maximum at 24 DAF, and thereafter it gradually decreased. There were no significant amounts of enzyme activities in other parts of the fruits. In Subicho (inbred line) the content of DHC was slightly higher than CAP in all parts of the fruits throughout the developmental stages of fruits, whereas in Chung Yang the CAP content was higher compared to the DHC content. The contents of total capsaicinoids in Chung Yang were also higher than Subicho. However, the crude enzyme extract obtained from Chung Yang led to the synthesis of DHC almost exclusively when the substrate, 8-methyl nonanoic acid, was added to the reaction mixture. Our results suggest that the composition of individual analogue of capsaicinoids depends upon the substrate available in the fruits.

Key words: *capsaicinoids, capsaicinoid synthetase,* Capsicum, *placenta, fruit.*

The pungency of pepper is due to capsaicinoids present in the fruits. CAP and DHC are the major analogues occupying more than 90% of the total capsaicinoid contents in fruits of most varieties of *Capsicum annuum*, whereas HC, HDC, and NDC are the minor analogues.[1] HPLC procedures were already developed to separate different capsaicinoid analogues.[2,3] The biosynthetic pathway leading to the capsaicinoid production has not been fully elucidated. Capsaicinoids have two distinct arms, one of which contributes to the fatty acid moiety and the other to the aromatic component.[4] They are end products of aromatic metabolic pathway that shares common precursors and intermediates with the phenylpropanoid pathway. This pathway leads to the formation of a wide range of plant metabolites like lignins, anthocyanins, coumarins, tannins, cell-wall phenolics, and flavonoids. The last enzyme, capsaicinoid synthetase, of the capsaicinoid biosynthetic pathway actually determines the accumulation of capsaicinoids in the fruits. This enzyme carries out the condensation of vanillylamine with the acyl moieties of fatty acid chain.[5] Since the capsaicinoids are synthesized exclusively in the placenta but accumulated mainly in the pericarps,[6-8] the main objective of this study was to correlate the capsaicinoid synthetase activity of placenta with the capsaicinoid contents of different parts of fruits at various physiological stages of *C. annuum* L. var. Subicho and Chung Yang, one of the highest pungent commercial cultivar in Korea.

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Abbreviations: CAP, capsaicin; CS, capsaicinoid synthetase; DAF, days after flowering; DHC, dihydrocapsaicin; HC, homocapsacin; HDC, homodihydrocapsaicin; HPLC, high pressure liquid chromatography; NDC, nordihydrocapsaicin.

Materials and Methods

The fresh fruits of *C. annuum* L. var. Subicho and a cv. Chung Yang (Choong Ang seed Co., Ltd.) were procured from the greenhouse of National Institute of Agricultural Science and Technology, RDA, Korea. During anthesis individual flowers of *Capsicum annuum* L. were tagged with the dates of flowering. The fruits of Subicho and Chung Yang were harvested in 12 day-intervals for a period of 58 days.

Extraction and assay of enzyme activity. All operations were carried out at 0 to 4°C. Different parts of the fruits viz. seeds, placenta, and pericarps were separated carefully and homogenized according to the method previously reported.[1] The tissues were homogenized with 6 volumes of extraction buffer (0.1 M Tris-HCl, pH 7.8, 10

mM β-mercaptoethanol). After removal of large tissue fragments by filtrating through a double layer of gauze, the filtrate was centrifuged at 12,800 × g for 20 min at 4°C. The supernatant was then used as the enzyme source, and the enzyme activity was determined immediately. Protein concentration was determined using the Bradfords method with bovine serum albumin as the standard.[9]

Assay method for the capsaicinoid synthesizing enzyme activity. CS activity was determined as previously described.[10] Enzyme activity for the formation of different capsaicinoids was assayed through HPLC in the enzyme extract before and after the addition of 4-hydroxy-3-methoxybenzylamine (vanillylamine) and 8-methyl nonanoic acid. The reaction mixture contained 0.025 m*l* of each 0.2 M vanillylamine, 40 mM ATP, 40 mM $MgCl_2$, 40 mM 8-methyl nonanoic acid, 40 mM CoA-SH, and 0.3 m*l* enzyme preparation. The final volume of the reaction mixture was made to 1 m*l* with 0.1 M Tris-HCl (pH 7.8). The reaction was performed at 37°C for 1 h and terminated with 0.1 m*l* of 12 N HCl. The assay mixture was extracted with 1 m*l* of chloroform, and the organic phase was evaporated to dryness at 50°C *in vacuo*. The residue was dissolved in 0.5 m*l* of HPLC grade acetonitrile, and capsaicinoids were determined through HPLC.

Analysis of capsaicinoid analogues. Different parts of fruits viz. placenta, pericarps, seeds, and whole fruits were oven-dried at 50°C for 2 to 4 days, ground in a mixer, and passed through a sieve (300 μm in diameter). All samples were processed within 7 days after harvesting. To the ground material (0.1 g), 5 m*l* of acetonitrile was added, and cyclomixing was done several times. The tubes were placed at room temperature overnight and then filtered. The acetonitrile was then evaporated under nitrogen gas, and 1 m*l* of HPLC grade acetonitrile was added to each sample. After the solution was microfiltered through 0.2-μm millipore filters (Nihon Millipore Kogyo K.K., Japan), 20 μ*l* of aliquot was used for each HPLC injection. A Shimadzu model LC-6AD HPLC equipped with a Waters Radial-Pak C18 column (5 μm, 8 mm × 10 cm) was used. The column was eluted isocratically with methanol : water (65 : 35, v/v) at the flow rate of 1.8 m*l*/min, and the detector was set at 280 nm.

Capsaicinoid standards. Standards of 8-methyl-*N*-vanillyl-6-nonenamide (CAP) and 8-methyl-*N*-vanillyl-nonanamide (DHC) were obtained from Sigma Chemical Co. (USA) and were used for retention time verification and instrument calibration. Solutions of 5, 10, 20, 30, 40, 50, and 100 PPM in methanol were used for making each standard curve. Quantification of capsaicinoids was achieved using the standard curve.

Results and Discussion

The contents of capsaicinoids (NDC, CAP, and DHC) of various parts of fruits viz. placenta, pericarps, seeds, and whole fruits harvested at different developmental stages were compared. The capsaicinoid contents in Subicho were detected as early as 12 DAF (Fig. 1A), but there was only slight increase in capsaicinoid contents up to 24 DAF. Significant increase was observed at 36 DAF, whereas the contents in the whole fruits slightly decreased at 48 DAF. Thereafter, capsaicinoid contents increased gradually throughout the fruit development examined. Fruits of Chung Yang had more capsaicinoid content at all developmental stages (Fig. 1B) as compared to the inbred line Subicho. A significant amount of capsaicinoids (1.0 mg/g) was already synthesized up to 12 DAF, and maximum increase (3.5 mg/g) was observed up to 36 DAF. Thereafter, there was a slight decrease in capsaicinoid contents in whole fruit when the fruit matured and the color of fruit changed (Fig. 1B). As the fruit-ripening proceeds, the color of fruit changes from green to red, pericarps and seeds harden, and several of other metabolites are synthesized. This suggests that there is a competition for the C-skeletons needed for the capsaicinoid biosynthesis and other metabolites.[11]

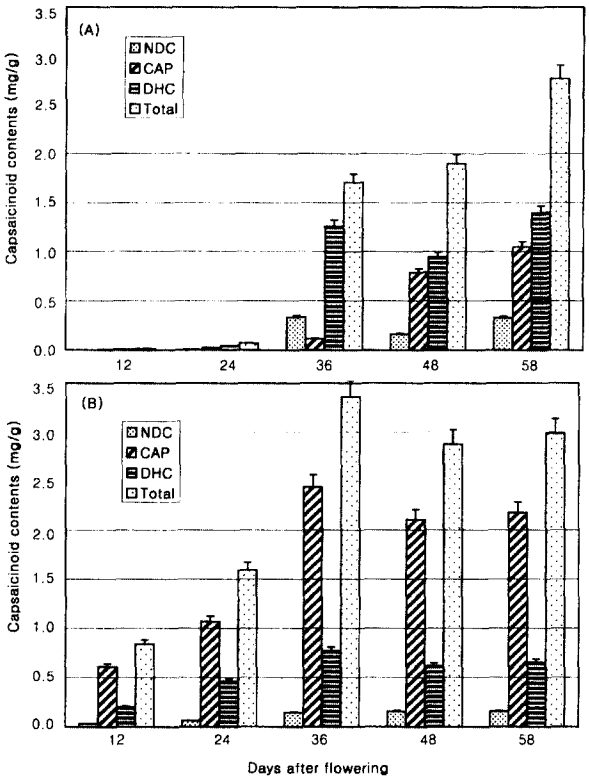

Fig. 1. Capsaicinoid contents of whole fruits. Individual and total contents of major capsaicinoids (NDC, nordihydrocapsaicin; CAP, capsaicin; DHC, dihydrocapsaicin) in whole fruits of *Capsicum annuum* L. var. Subicho (A) and cv. Chung Yang (B) were examined at various stages of fruit development (12, 24, 36, 48, and 58 DAF).

In general, Chung Yang contained higher level of total capsaicinoids (3.5 mg/g) as compared to the inbred line Subicho (2.5 mg/g). In further detailed analysis of the individual analogue of capsaicinoids, the CAP content was

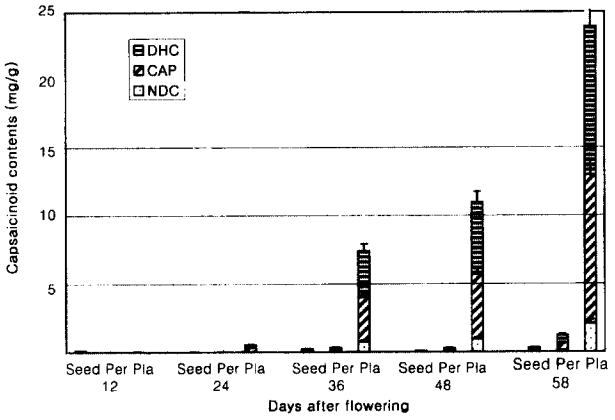

Fig. 2. Capsaicinoid contents in different parts of fruits. Capsaicinoid contents in different parts of fruits viz. seeds, pericarps (per), and placenta (pla) of *Capsicum annuum* L. var. Subicho were examined at various stages of fruit development.

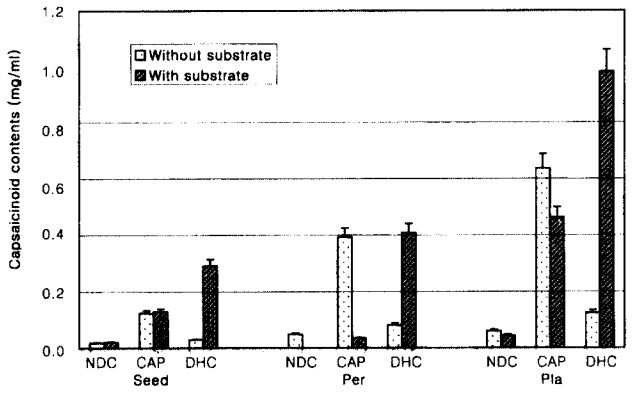

Fig. 3. Substrate specificity of crude capsaicinoid synthetase enzyme extract from *Capsicum* fruits. Crude extracts from seeds, pericarps (per), and placenta (pla) of Chung Yang fruits were added to the enzyme reaction mixture along with 8-methylnonanoic acid, and the analogues of capsaicinoids (NDC, Nordihydrocapsaicin; CAP, Capsaicin; DHC, Dihydrocapsaicin) were analyzed after 1 h incubation. In the control, no substrates were added.

found to be much higher in Chung Yang (over 60% of total capsaicinoids), whereas the DHC content was predominant in Subicho (almost 50% of total capsaicinoids) at all stages (Figs. 1A and 1B). CAP and DHC are known as the main analogues of capsaicinoid in fruit,[1] and CAP is the main component responsible for the pungency of *Capsicum* sp..[12] Chung Yang seemed to be more pungent compared to Subicho. Indeed, Chung Yang is known as one of the most pungent commercial cultivars in Korea.

In order to compare the contents of individual analogues of capsaicin in different organs of *Capsicum* fruits, HPLC analysis was carried out to estimate the capsaicinoid content of the placenta, seeds, and pericarps. The total capsaicinoid contents were very low up to 24 DAF; thereafter, they increased drastically in all parts of the fruits of Subicho (Fig. 2). The capsaicinoid contents were maximum in placenta (23.9 mg/g dry wt.) followed by whole fruits (2.77 mg/g dry wt.) and pericarps (1.21 mg/g dry wt.) and minimum in seeds (0.309 mg/g dry wt.) of mature fruits at 58 DAF. The DHC contents were higher than the CAP contents in placenta, whole fruits, and pericarps. However, in seeds the DHC was slightly less than the CAP at all stages of the fruit development, although the actual amount was too small to differentiate (Fig. 2).

The CS activity was compared with the capsaicinoid accumulation in different parts of fruit at different developmental stages. It was observed that the product of CS in the crude extracts from placenta was DHC when the substrate was used 8-methyl nonanoic acid.[1] The crude extracts obtained from both Subicho and Chung Yang produced a high amount of DHC as the final reaction product as previously reported.[1] In particular, the enzyme extract of Chung Yang that contained a higher amount of CAP indigenously also yielded a large amount of DHC (1.0 mg/m*l*) when incubated for 1 h after adding vanillylamine and 8-methyl nonanoic acid (Fig 3). These results indicate that the product of this enzyme depends upon the available

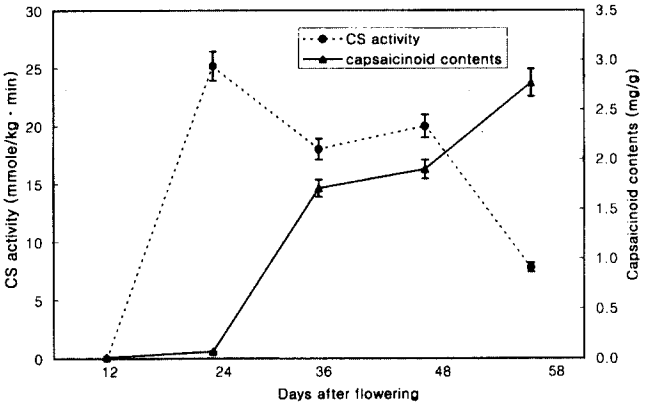

Fig. 4. The activity profile of CS at different stages of fruit development. CS activity (mmole/kg · min) with respect to capsaicinoid accumulation (mg/g of dry weight) in the placenta of *Capsicum annuum* L. var. Subicho was examined at various stages of fruit development.

substrate and Chung Yang may not supply enough amount of 8-methyl nonanoic acid (iso-$C_{10:0}$). Availability of fatty acid chain is also suggested to determine the final analogue of capsaicinoids, but no conversion occurs after the condensation with the vanillylamine moiety. It has been reported that valine is incorporated into CAP and DHC, which have even numbers of the branched acyl chains, while leucine is incorporated into NDC, HDC, and HC, which have odd-numbered branched chain fatty acids as the acyl moieties.[13] Capsaicinoid compounds possessing odd-numbered branched fatty acids are rare in the *Capsicum* fruits, although they were observed in *in vitro* experiments with the addition of various substrates such as 7-methyl octanoic acid and 9-methyl decanoic acid.[1] The substrate for CAP that has even-numbered branched fatty acid was also determined as 8-methyl-6-nonenoic acid.[5] Taken together, our results suggest that there is much less endogenous

substrate for, 8-methyl nonanoic acid, DHC in Chung Yang compared to Subicho.

During fruit deveplopment, CS activity in placenta, the main site of synthesis of the capsaicinoids synthesis, increased to maximum (at 24 DAF) and then decreased (Fig. 4). The highest activity was observed at 24 DAF (25 mmoles of DHC formed/kg protein · min) and decreased slightly at 36 DAF (18 mmoles of DHC formed/kg protein · min). Thereafter, a slight increase in activity was observed at 48 DAF, which again decreased significantly. In the beginning when the size of fruit was increasing, all the precursors were diverted to primary metabolism and were not available for capsaicinoid synthesis.[14] When the maximum size of fruit was achieved, the capsaicinoid contents started accumulating. Taken together a probable relationship might exist between the age/the length of fruit and the accumulation of capsaicinoids. We also observed that the periodic changes capsaicinoid contents in placenta (Fig. 2 and Fig. 4) is well related with those of the enzyme activities. Purification of the CS enzyme and isolation of the gene encoding this enzyme are now in progress.

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