Note

The Precursors Affecting the Composition of Capsaicin and Its Analogues in the Fruits of *Capsicum annuum* var. *annuum* cv. Karayatsubusa

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With regard to the pungent principle of red pepper, capsaicinoid, at least five compounds have been reported in nature: capsaicin, dihydrocapsaicin, nordihydrocapsaicin, homodihydrocapsaicin and homocapsaicin, the structures of which are vanillyl amides of 8-methylnon-*trans*-6-enoic acid, 8-methylnonanoic acid, 7-methylnonanoic acid, 9-methyldecanoic acid and 9-methyldec-*trans*-7-enoic acid, respectively.^{1~3)} Among them, CAP and DC are the major components of the majority of *Capsicum* species. On the biosynthesis of fatty acyl moieties of capsaicinoid, Leete and Loudon⁴⁾ suggested that L-valine serves as the precursor for the formation of even-number branched chain fatty acyl moieties of CAP and DC. However, the precursor of the biosynthesis of odd-number fatty acyl moieties of NDC, HDC and HC has not been revealed yet. Recently, authors found that L-leucine serves as the precursor of odd-number branched chain fatty acyl moieties of NDC, and HDC.⁵⁾

In nature, the actual content of NDC is only in a small amount, and that of HDC and HC is usually negligible. Why are capsaicinoid analogues having odd-number branched chain fatty acyl moieties so little? Are they regulated by a pool of free amino acids and/or by any other mechanism? On the amino acids of *Capsicum* fruit, there have been few studies, except one paper dealing with the viewpoint of food chemistry.⁶⁾ No report has been presented in respect to capsaicinoid formation and accumulation during maturation of the fruit. The present

paper is a preliminary work to examine the relationship between the compositions of capsaicinoid and free amino acids in the placenta of *Capsicum* fruit during maturation after flowering.

The red pepper fruit used was *Capsicum annuum* var. *annuum* cv. Karayatsubusa cultivated and harvested on our experimental farm from August through September, 1978. The fruits were collected every ten days from 15 through 45 days after flowering. After removal of seeds, the placentas were separated and stored at -80°C until use. Free amino acids in the placenta were extracted with several volume of hot 75% ethanol three times. After evaporating off the alcohol, the alcohol extract was washed with ether after a filtration through a Millipore filter. Samples thus prepared were stored below -20°C until the amino acid analysis. The amino acids were analyzed by a Hitachi KLA-5 amino acid analyzer equipped with Shimadzu Chromatopac E-1A micro-computer.

Capsaicinoid was extracted with 10 volumes of hot isopropanol, 5 volumes of chloroform-isopropanol (1:1, v/v), and finally 5 volumes of chloroform. To remove pigments, the crude capsaicinoid extract was first chromatographed on a precoated silica gel G plate (250μ thick, E. Merck, Darmstadt) using petroleum ether-ethyl ether-acetic acid (60:40:1, v/v/v). The zone corresponding to capsaicinoid was scraped and extracted with ethyl acetate. The ethyl acetate solution containing capsaicinoid was spotted on the precoated silica gel G plate (250μ thick, E. Merck, Darmstadt) and developed with toluene-chloroform-acetone (40:35:25, v/v/v) to separate the capsaicinoid. Quantification of the total capsaicinoid content was performed by Shimadzu dual wavelength TLC scanner, CS-910. The wavelengths were set at 280 nm for the sample side and 350 nm for the reference side. Another amount of ethyl acetate solution containing capsaicinoid was subjected to mass chromatography after being modified to trimethylsilyl derivatives. Determination of the capsaicinoid composition was done by mass chromatography established in our laboratory.⁷⁾

Capsaicin and its analogues can be classified into 2 groups on the basis of the chainlengths of their branched chain fatty acyl moieties: Namely, CAP and DC with even-number branched chain fatty acyl moieties (*Capsaicinoid group 1*); NDC, HDC and HC with odd-number branched chain fatty acyl moieties (*Capsaicinoid group 2*). Since capsaicinoid is formed and accumulated almost exclusively in the placenta,⁸⁾ the capsaicinoid composition in the placenta at different stages after flowering was analyzed. Capsaicinoid was first detected on 15 days after flowering.

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Abbreviations: CAP, capsaicin [*N*-(4-hydroxy-3-methoxybenzyl)-8-methylnon-*trans*-6-enamide]; DC, dihydrocapsaicin [*N*-(4-hydroxy-3-methoxybenzyl)-8-methylnonamide]; NDC, nordihydrocapsaicin [*N*-(4-hydroxy-3-methoxybenzyl)-7-methyloctamide]; HDC, homodihydrocapsaicin [*N*-(4-hydroxy-3-methoxybenzyl)-9-methyl-dec-*trans*-7-enamide]; HC, homocapsaicin [*N*-(4-hydroxy-3-methoxybenzyl)-9-methyldec-*trans*-7-enamide].

The content of capsaicinoid reached its maximum around 25 days after flowering, then decreased gradually. As for the capsaicinoid composition, more than 70% was occupied by CAP, 29% by DC and 1% by NDC in the placenta of 15 days after flowering. Similarly, 52 to 55% was occupied by CAP, 44 to 41% by DC, 3 to 4% by NDC, and 0.1 to 0.2% by HDC in the placenta of 25 days after flowering. It is obvious that *Capsaicinoid group 1* is the major component occupied more than 95% of the total capsaicinoid. On the other hand, as for *Capsaicinoid group 2*, NDC occupied less than 5%, and HDC and HC were hardly detected.

Table I shows the molar concentration of L-valine, L-leucine and L-phenylalanine in the placenta at different stages after flowering. L-Phenylalanine serves as the precursor of vanillylamine moiety. The molar concentration of free L-valine in the placenta reached its maximum at 25 days after flowering, then decreased after that. The content of free L-leucine also showed a similar pattern to that of L-valine, but was less than the level of L-valine at any stage examined. It seems that the fluctuation of capsaicinoid composition and content is related to those of valine and leucine.

From the results in Table I, if there exists a free amino acid pool for the branched chain fatty acids, it can be suggested that the L-valine pool size is greater than that of L-leucine at any stage after flowering in the placenta. As shown in Table II, the isotope labeled free amino acids, L-[U- 14 C] valine and L-[U- 14 C] leucine, were incorporated into capsaicinoid molecules. The fact that L-[U- 14 C] leucine was more effectively incorporated into capsaicinoid moiety (HDC) without any significant dilution of the specific activity can be an evidence of the smaller leucine pool size for the *Capsaicinoid group 2*. It is not amenable that the capsaicinoid composition is regulated by the substrate specificity of the enzyme which catalyzes the condensation reaction of the capsaicinoid formation from vanillylamine and various branched chain fatty acyl CoAs.⁹⁾ Therefore, the difference of the capsaicinoid composition in the *Capsicum* fruit may be attributed to the

TABLE I. CONTENT OF FREE VALINE, LEUCINE AND PHENYLALANINE IN THE PLACENTA OF *Capsicum* FRUIT AFTER FLOWERING

Free amino acids were extracted by the procedure described in the text, and analyzed by an amino acid analyzer. Values are expressed as $\mu\text{mol/g}$ of dry placenta, and they are mean values obtained from triplicated experiments using five fruits for each stage.

Amino acid	Days after flowering			
	15	25	35	45
L-Valine	0.96	1.29	0.41	0.32
L-Leucine	0.13	0.24	0.11	0.10
L-Phenylalanine	trace	0.11	0.17	0.33

difference in the placenta of each of free amino acid content which serve as the precursor of the branched chain acyl moieties.

Another factor which affects the capsaicinoid composition may be a diglyceride. The radioactivity only in the diglyceride fraction rapidly increased after feeding the placenta ^{14}C -valine and ^{14}C -leucine, then decreased in company with the increase of the radioactivity in the capsaicinoid fraction. On the other hand, neither appreciable amount of radioactivity nor branched chain fatty acid could be detected in the free fatty acid, phospholipid, triglyceride and sterol ester fractions at any stage during the incubation. Detailed studies on the species and size of the amino acid pool, and fluctuation of diglyceride content and composition during the capsaicinoid biosynthesis are now in progress.

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TABLE II. INCORPORATION OF L-[U- 14 C] VALINE AND L-[U- 14 C] LEUCINE INTO THE CAPSAICINOID MOIETIES IN THE PLACENTA OF *C. annuum* var. *annuum* cv. KARAYATSUBUSA FRUITS

In both experiments, 5 μCi each of L-[U- 14 C] valine or L-[U- 14 C] leucine was separately fed *via* the stem of "Karayatsubusa" plant using defatted cotton thread.⁸⁾ Feeding was carried out for 5 days at 25° to 30°C in the green house. Six fruits were collected to analyze the radioactivity in capsaicinoid. Other experimental conditions were essentially the same as to those in the previous paper.⁸⁾ Values are expressed as the measured cpm by a liquid scintillation spectrometer and specific activity.

Amino acid fed	Fresh placenta (g)	Radioactivity in capsaicinoid	
		(detected cpm)	(cpm/g capsaicinoid)
L-[U- 14 C] valine	0.70	3547	168,900
L-[U- 14 C] leucine	0.45	3794	281,040

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