Simple Assay Method for Determination of Capsaicinoid Synthetase Activity

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A new method to assay the capsaicinoid synthetase (CS) activity was developed by utilizing NADH-coupled enzyme systems involving pyruvate kinase and lactate dehydrogenase. CS activities in *Capsicum* placenta, depending upon the kinetics of the NADH oxidation, revealed almost the same profile as compared with those shown using an HPLC-based method. When the substrates, 8-methyl nonanoic acid and vanillylamine, for the CS enzyme were employed separately or simultaneously, it appeared that the two-step reaction, acyl-CoA formation and condensation with vanillylamine, of the CS enzyme was a coupled reaction. Thus, this assay method of the CS enzyme can be considered as an alternative to the HPLC-based method, since it has the advantages of rapidity and simplicity as well as reliability when compared with the existing method.

Key words: *capsaicinoid synthetase,* Capsicum annuum, *lactate dehydrogenase, NADH, pyruvate kinase.*

The pungent principles of chilli pepper fruits are alkali labile esters, capsaicinoids, of which CAP and DHC are the main components (more than 90%).[1] Although a probable metabolic pathway leading to capsaicinoids has been previously suggested,[2,3] the pathway has not been fully elucidated until now. CS catalyzes the last step of the biosynthetic pathway, which carries out the enzymatic condensation of vanillyamine with acyl moiety of various acyl-CoAs.[4-7] There are several different methods available for determination of the CS activity.[8,9] However, the existing methods are tedious and time-consuming, because a considerable effort is required for the extraction of reaction products with organic solvents. Furthermore, the products have to be analyzed using HPLC. Therefore, it is necessary to develop an alternative, simple method for the assay of CS activity.

Pyruvate kinase (EC 2.7.1.40) and lactate dehydrogenase (EC 1.1.1.27) have been extensively employed for the activity assay of diverse enzymes consuming ATP as an energy source or a phosphate donor. The resulting ADP is reverted to ATP by receiving a phosphate group from PEP, whereby PEP is converted to pyruvate, which, in turn, is used as a substrate of lactate dehydrogenase that oxidizes NADH to NAD^+. Therefore, the amount of ADP produced by the CS activity (Fig. 1) can be estimated via a spectrophotometric measurement of the NADH oxidation; light absorption of NADH at 340 nm decreases as NADH is oxidized into NAD^+.

We report here an alternative, new method for the assay of CS activity. The simplicity and reliability of our method is also discussed through comparison with the existing HPLC-based method.

Materials and Methods

Plant materials. *Capsicum annuum* var. Subicho was grown in the green house of NIAST, RDA (Suwon). The fruits were harvested at 25 days after flowering. The placenta were then separated with a blade and immediately frozen in liquid nitrogen.

Extraction of CS enzyme. Unless indicated otherwise, all experiments were carried out at 0 to 4°C in a cold room. The basic enzyme extraction buffer contained 100 mM Tris-HCl buffers (pH 7.8) and 10 mM β-mercaptoethanol. The placenta was homogenized with this extraction buffer using a mortar and pestle. The homogenate was centrifuged at 12,850×g for 20 min at 0°C. The supernatant was collected and stored at 4°C for 7 days, and then used as the crude enzyme extract.

HPLC-based assay of CS activity. Under the standard assay condition as described,[7] the reaction mixture contained 575 μl of 100 mM Tris-HCl (pH 7.8) and 25 μl each of 0.2 M vanillylamine, 40 mM 8-methylnonanoic acid,

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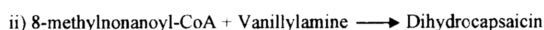
Abbreviations: CAP, capsaicin; CS, capsaicinoid synthetase; DAF, days after flowering; DHC, dihydrocapsaicin; FPLC, fast performance liquid chromatography; HPLC, high pressure liquid chromatography; NDC, nordihydrocapsaicin; PEP, phosphoenol pyruvate.

A. Two Reaction Steps of Capsaicinoid Synthetase (CS)



B. Schematic representation of NADH-coupled method

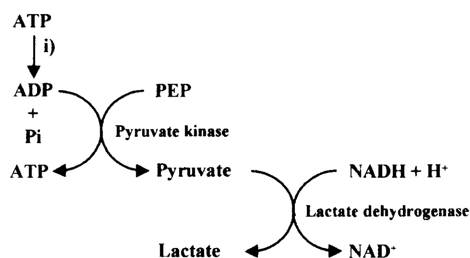


Fig. 1. Strategy for assay of capsaicinoid synthetase. (A) A proposed model of two-step reaction of the CS enzyme. Dihydrocapsaicin, one of the abundant capsaicinoids, is formed via two-step reactions, acyl-CoA formation and condensation with vanillylamine. (B) An ADP from the first reaction is reverted to ATP by the action of pyruvate kinase, in which PEP is converted to pyruvate. The pyruvate is then reduced to lactate by lactate dehydrogenase, whereby NADH is oxidized to NAD^+. The kinetics of NADH oxidation are used for the calculation of the CS activity (first reaction).

40 mM CoA-SH, 40 mM ATP, and 40 mM $MgCl_2$. The reaction was initiated by the addition of 300 μ*l* of crude enzyme extracts prepared as above. The reaction was performed at 37°C for 1 h and terminated by the addition of 0.1 m*l* of 12 N HCl. Subsequently, the assay mixture was extracted with 1 m*l* of chloroform. The organic phase was then carefully collected and evaporated to dryness at 50°C *in vacuo*. The residue was dissolved in 0.5 m*l* of HPLC-grade acetonitrile and filtered using a 0.2 μm Millipore membrane (Nihon Millipore Kogyo). Concentration of the capsaicinoids were determined using HPLC (LC-6AD, Shimadzu) equipped with a μBonda-Pak C_{18} column (5 μm in diameter, 0.8 cm×10 cm). 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. The standard reagents, 8-methyl-*N*-vanillyl-6-nonenamide (CAP) and 8-methyl-*N*-vanillyl-nonamide (DHC), were obtained from Sigma Chemical Co. (USA) and were used for retention time verification, instrument calibration, and quantification of the samples.

NADH-coupled assay of CS activity. We developed an NADH-coupled method to measure the CS activity on the basis of the kinetics of NADH oxidation.[10,11] The reaction mixture (975 μ*l*) consisted of 25 μ*l* each of 40 mM CoA-SH, 40 mM ATP, 40 mM $MgCl_2$, 50 mM KCl, 1 mM sodium azide, 0.5 mM sodium orthovanadate, 1 mM sodium molybdate, 200 mM potassium nitrate, 0.2 mM PEP, 2 units (5 μ*l*) of pyruvate kinase/lactate dehydrogenase (mixed form, Sigma), and 0.1 m*l* of enzyme preparation. None, both or either of the substrates (0.2 M vanillylamine and 40 mM 8-methyl nonanoic acid) for the CS enzyme was added to the reaction. After placing the tube at 37°C for 5 min, 25 μ*l* of 0.2 mM NADH was added to start the reaction. The rates of NADH oxidation at different time intervals were measured at 340 nm with a UV/VIS spectrophotometer (BioSpec-1601, Shimadzu, Japan). Since the sample solution may contain endogenous substrates for capsaicinoids synthetase or other ATP-hydrolyzing enzyme, CS activity was calculated by subtracting NADH oxidation rate of the blank from that of the sample.[12] Proteins were quantified using Bradford's method with bovine serum albumin as the standard.[13]

Chromatography. The approximate molecular weight of the CS enzyme was determined by separately applying the crude enzyme extract and the protein standards (Gel filtration standard, Bio-Rad Co.) to a Sephadex LH-20 column (pre-equilibrated with 0.1 M Tris-HCl, pH 7.8) on the FPLC system (Pharmacia Co.).

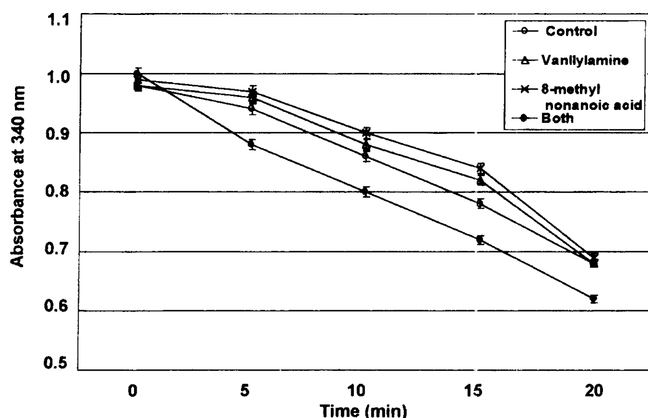


Fig. 2. Absorbency profiles in the NADH-coupled reaction mixtures in the presence or absence of different combinations of substrates for the CS enzyme. No substrate, control (○); single substrate, vanillyliamine (Δ) or 8-methyl nonanoic acid (X); both substrates, vanillylamine and 8-methylnonanoic acid (●).

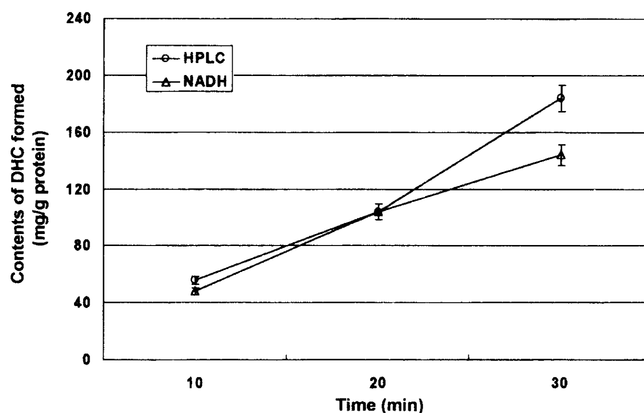


Fig. 3. Comparison of the NADH-coupled reaction with that of the HPLC-based method. The amount of DHC formed from each reaction of the two methods was determined via HPLC using capsaicinoid standards. HPLC, reaction of HPLC-based method; NADH, NADH-coupled reaction.

Table 1. CS activities measured using the NADH-coupled and HPLC-based methods (end-product determination). CS activities of each method were calculated by determining the amount of end-product, DHC, via HPLC analysis.

Methods	CS activity
HPLC-Based method (μmoles of DHC formed/min · mg)	0.018 ± 0.002
NADH-coupled method (μmoles of NADH oxidized/min · mg)	0.038 ± 0.004

Results and Discussion

Comparison of the two assay methods. Previously, the CS activity has been determined by measuring the amount of end-products of the reaction using a radio-labeled substrate[1] or by quantifying the components of the product via mass spectrometry following thin-layer and gas liquid chromatographies.[3,8] However, these methods are labor-intensive and time-consuming. In our present study, we tested a new assay method for the CS activity by coupling the production of ADP with enzymatic NADH oxidation through consecutive actions of pyruvate kinase-lactate dehydrogenase. In the previous studies, [10,11] measurement of the ATP hydrolytic activities has been successfully done using the coupled NADH oxidation. To exclude the possibilities of involvement of other ATP hydrolytic activities that may contribute to the production of ADP, sodium azide, sodium orthovanadate, and potasium nitrate were added to the reaction mixture.

The CS catalyzes the condensation of 8-methyl nonanoic acid with vanillylamine to form DHC. It has been proposed that this reaction might take place in two steps; the enzymatic conversion of 8-methyl nonanoic acid to its CoA form by consuming ATP, and the condensation of the acyl-CoA with vanillylamine to yield a DHC.[1] In the enzyme coupled assay, ADP produced during the first step serves as a substrate for pyruvate kinase that converts phosphoenol pyruvate and ADP to pyruvate and ATP, respectively. Lactate dehydrogenase then oxidizes NADH at the expense of pyruvate, which is monitored through the absorbency change at 340 nm (Fig. 1). When the components of the enzyme-coupling system were added to the CS reaction mixture, the ABS_{340} decreased as the reaction proceeded (Fig. 2). This result indicates that the ADP production during the CS-catalyzed reaction is coupled to NADH oxidation as expected. However, considerable NADH oxidation was also observed in the absence of either one or both of the substrates. This substantial NADH oxidation may be contributed either to the presence of an unknown ATP-hydrolyzing activity or to precursors of capsaicinoids contaminated during the sample preparation. At present, the unidentified ATP-hydrolyzing activity may be a more plausible explanation for such a substantial decrease in ABS_{340} without CS substrates, since the NADH oxidation rate without CS substrates remained steady throughout the reaction period. Nevertheless, difference in NADH oxidation rates between the presence and the absence of the substrates was sufficient for the calculation of CS activity.

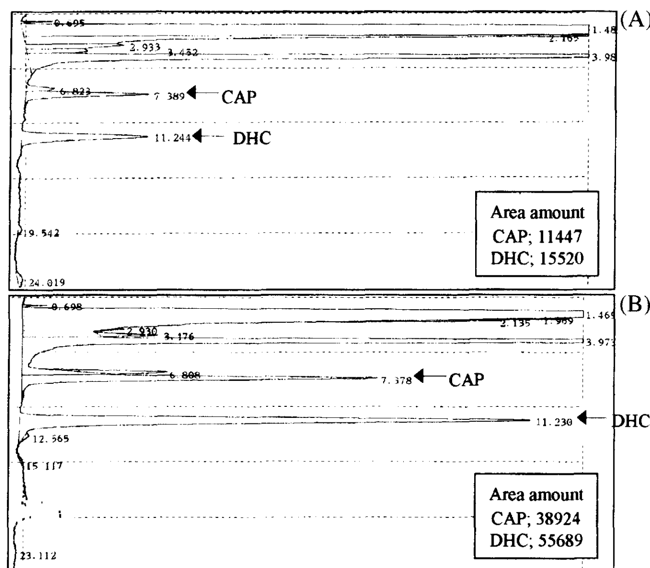


Fig. 4. Chromatogram of capsaicinoids from HPLC analysis. Capsaicinoids were analyzed using HPLC system before (A) and after (B) enzyme reaction for 1 h. Twenty micro-liter of each sample was loaded into the column.

In order to examine whether the addition of the enzyme-coupling system to the CS assay mixture affects the capsaicinoid synthesizing reaction, the amount of DHC produced from the CS reaction in the presence of the coupling system was compared to that of the uncoupled reaction condition (Fig. 3). After 10, 20 and 30 min from the initiation of the reaction, the amount of DHC in the reaction mixtures was quantified using the HPLC method. Plots of the reaction time versus DHC production under both conditions were similar to each other, indicating that the enzyme coupling system, such as pyruvate kinase, lactate dehydrogenase, PEP, and NADH, did not interfere with the CS reaction. However, when the CS activity was measured via HPLC-based product analysis and spectroscopic-monitoring NADH oxidation, the enzyme coupled-assay method yielded higher CS activity than the DHC analysis method (Table 1). The CS activities measured through the enzyme-coupled method and DHC analysis were 0.038 ± 0.004 and 0.018 ± 0.002 μmole/min · g protein, respectively. What causes such difference in the calculated CS activity between the two assay methods? One of the probable causes for such difference would be the synthesis of by-products during the reaction. In the presence of 8-methyl nonanoic acid and vanillylamine as substrates, the expected product of CS-catalyzed reaction is DHC. However, an increase in a considerable amount of other capsaicinoids was also detected (Fig. 4).[1] In particular, increase in CAP was significant, indicating that a precursor of CAP may exist in

Table 2. CS activities of placenta at different developmental stages of fruit measured using the NADH-coupled and HPLC-based methods (end production determination).

Stage(DAF)	NADH method (μmoles of NADH oxidized/min·mg)	HPLC-Based method (μmoles/min·mg)		
		DHC	CAP	SUM
12	0.003 ± 0.002	ND	ND	ND
24	0.043 ± 0.005	0.022 ± 0.006	0.024 ± 0.003	0.046 ± 0.009
36	0.038 ± 0.004	0.018 ± 0.002	0.016 ± 0.002	0.034 ± 0.004
48	0.039 ± 0.003	0.018 ± 0.002	0.014 ± 0.005	0.032 ± 0.007
60	0.009 ± 0.002	0.007 ± 0.002	0	0.007 ± 0.002

ND, Not detected.

the assay mixture. Possible source of such contamination would be the sample solution, since crude extract from developing pepper fruits was used in this study. Based on this observation, we calculated CS activity using the HPLC measurement by summing the amount of DHC and CAP as CS reaction products, and compared it to that from the spectroscopic measurement (Table 2). For the CS preparations from five different stages of pepper-fruit development, comparable CS activities were calculated via both measurements. CS activity, 0.003 ± 0.002 μmoles/min · g protein, was minimal at the very early stage of the fruit development (12 DAF) as determined using the spectroscopic method. However, CS activity could not be calculated through the HPLC-based method since the amount of products was too low. At 24 DAF, CS activities calculated from ΔABS_{340} and the product quantification were 0.043 ± 0.005 and 0.046 ± 0.009 μmole/min · g protein, respectively. With further progress, CS activity decreased gradually as calculated using both methods of CS assay.

When the NADH-coupled method was employed for the CS assay, we found that the CS activity depended upon the protein concentration in the crude enzyme extract (data not shown). As the protein concentration of the crude enzyme extracts increased up to 6.3 mg/m*l*, the activity continually increased. However, it thereafter decreased as the concentration of protein increased. We are currently attempting to purify the CS enzyme by measuring the activity using the NADH-coupled method. As a preliminary result, we observed that the CS enzyme might have a molecular weight of 100 kD, when crude enzyme extracts and the protein standards were applied separately to Sephadex LH-20 column on FPLC system. Measurement of ATP hydrolyzing activity by the enzyme-coupled method is fast, and result with high reliability compared to HPLC-based methods currently practiced in many laboratories can be obtained easily. This is especially important for the purification of enzyme.

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