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See next page for additional authors

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Functional Analysis of the Amine Substrate Specificity Domain of Pepper Tyramine and Serotonin N-Hydroxycinnamoyltransferases

Sei Kang, Kiyoon Kang, Gap Chae Chung, Doil Choi, Atsushi Ishihara, Dong-Sun Lee, and Kyoungwhan Back

Department of Molecular Biotechnology, Agricultural Plant Stress Research Center, Biotechnology Research Institute, Chonnam National University, Gwangju 500–757, Korea (S.K., K.K., G.C.C., K.B.); Korea Research Institute of Bioscience and Biotechnology, Daejeon 305–600, Korea (D.C.); Division of Applied Life Sciences, Graduate School of Kyoto University, Kyoto 606–8502, Japan (A.I.); and Structural Biology Center, Department of Biochemistry and Molecular Biology, University of Texas Medical School, Houston, Texas 77030 (D.-S.L.)


Pepper (Capsicum annuum) serotonin N-hydroxycinnamoyltransferase (SHT) catalyzes the synthesis of N-hydroxycinnamic acid amides of serotonin, including feruloylserotonin and p-coumarylserotonin. To elucidate the domain or the key amino acid that determines the amine substrate specificity, we isolated a tyramine N-hydroxycinnamoyltransferase (THT) gene from pepper. Purified recombinant THT protein catalyzed the synthesis of N-hydroxycinnamic acid amides of tyramine, including feruloyltyramine and p-coumaryltyramine, but did not accept serotonin as a substrate. Both the SHT and THT mRNAs were found to be expressed constitutively in all pepper organs. Pepper SHT and THT, which have primary sequences that are 78% identical, were used as models to investigate the structural determinants responsible for their distinct substrate specificities and other enzymatic properties. A series of chimeric genes was constructed by reciprocal exchange of DNA segments between the SHT and THT cDNAs. Functional characterization of the recombinant chimeric proteins revealed that the amino acid residues 129 to 165 of SHT and the corresponding residues 125 to 160 in THT are critical structural determinants for amine substrate specificity. Several amino acids are strongly implicated in the determination of amine substrate specificity, in which glycine-158 is involved in catalysis and amine substrate binding and tyrosine-149 plays a pivotal role in controlling amine substrate specificity between serotonin and tyramine in SHT. Furthermore, the indisputable role of tyrosine is corroborated by the THT-F145Y mutant that uses serotonin as the acyl acceptor. The results from the chimeras and the kinetic measurements will direct the creation of additional novel N-hydroxycinnamoyltransferases from the various N-hydroxycinnamoyltransferases found in nature.

N-Hydroxycinnamic acid amides (HCAAs), which are present in a diverse array of plant species, have important roles in plant-plant (Martin-Tanguy and Negrel, 1987; Cutillo et al., 2003), plant-pathogen (Clarke, 1982), plant-insect (Lajide et al., 1995), and plant-environment (Negrel et al., 1993; Schraudner et al., 1993) interactions. HCAAs are synthesized by the condensation of hydroxycinnamoyl-CoA thioesters and aromatic amines (Fig. 1). The hydroxycinnamoyl-CoA thioesters include cinnamoyl-CoA, p-coumaryl-CoA, caffeoyl-CoA, feruloyl-CoA, and sinapoyl-CoA, and are synthesized from cinnamic acid by a series of enzymes, including cinnamate-4-hydroxylase, coumarate-3-hydroxylase, caffeic acid O-methyltransferase, ferulate-5-hydroxylase, and hydroxyccinnamate:CoA ligase (Douglas, 1996). Aromatic amines are generated from Tyr, Trp, and dihydroxyphenylalanine by decarboxylation or hydroxylation and include tyramine, octopamine, tryptamine, serotonin, dopamine, and noradrenaline (Wink, 1997). HCAAs represent an important class of antioxidant and chemotherapeutic agents (Zhang et al., 1996; Kawashima et al., 1998; Nagatsu et al., 2000; Park and Schoene, 2002). The biosynthesis of the HCAAs of tyramine is catalyzed by tyramine N-hydroxycinnamoyltransferase (THT; EC 2.3.1.110). This reaction has been well characterized in investigations of the mechanisms that regulate HCAA synthesis, THT activity, and the absolute levels of enzymes, in comparison with other classes of N-hydroxycinnamoyltransferases, such as anthranilate N-hydroxycinnamoyl/benzoyltransferase (EC 2.3.1.144; Yang et al., 1997) and agmatine N-hydroxycinnamoyltransferase (EC 2.3.1.110).
Alignments of the amino acid sequences of these \( N \)-hydroxycinnamoyltransferases have revealed no significant similarities. Numerous THT enzymes from plant sources have been partially or completely purified and characterized (Farmer et al., 1999; Schmidt et al., 1999; Yu and Facchini, 1999; Ishihara et al., 2000). These enzymes behave as soluble proteins with a molecular mass of 26 to 28 kD. The deduced THT amino acid sequences, derived from cDNA sequences from potato (\textit{Solanum tuberosum}), tobacco (\textit{Nicotiana tabacum}), and tomato (\textit{Lycopersicon esculentum}), show a high degree of amino acid sequence identity, ranging from 73% to 76%. Mechanistic studies have shown that all of the THT enzymes exhibit similar substrate affinity patterns, especially at the level of amine substrates, with the exception of the maize (\textit{Zea mays}) enzyme (Ishihara et al., 2000). THT enzymes isolated from potato, tobacco, and tomato show the highest affinity for tyramine and octopamine (\( \beta \)-hydroxytyramine). Recently, a closely related \( N \)-hydroxycinnamoyltransferase gene was isolated from pepper (\textit{Capsicum annuum}; Jang et al., 2004). This \( N \)-hydroxycinnamoyltransferase fused with a His tag was expressed in \textit{Escherichia coli} and purified to homogeneity. The enzyme has a 16-fold lower \( K_m \) for serotonin (73 \( \mu \)M) than for tyramine (1,165 \( \mu \)M) with feruloyl-CoA as the acyl donor, revealing serotonin \( N \)-hydroxycinnamoyltransferase activity (SHT). The subsequent ectopic expression of pepper SHT in rice (\textit{Oryza sativa}) induced the production of large amounts of HCAAs of serotonin, such as feruloylserotonin and \( p \)-coumaroylserotonin, in transgenic rice, verifying in vivo that SHT is a true serotonin \( N \)-hydroxycinnamoyltransferase (Jang et al., 2004; Kang et al., 2005). In spite of considerable progress in the study of \( N \)-hydroxycinnamoyltransferases, at both the molecular and substrate specificity analysis levels, a more detailed study of the residues and domains required for substrate binding and enzyme activity has not been conducted.

In this study, we attempted to determine the regions or the key residues of \( N \)-hydroxycinnamoyltransferase that are required for amine binding and enzyme activity, using pepper \( N \)-hydroxycinnamoyltransferases. We first isolated from pepper a THT that is not able to use serotonin as an acyl acceptor. Because of both the high level of amino acid identity between SHT and THT (78%) and a more or less equal distribution of amino acid substitutions and mismatches throughout the deduced amino acid sequences (Fig. 2A), we were not able to readily identify domains that might contribute to the amine-binding specificity domain. Therefore, a functional analysis using chimeric proteins constructed from the SHT and THT sequences

![Figure 1. Schematic diagram of the enzymatic reaction catalyzed by THT and SHT. Biosynthesis from Trp through serotonin is catalyzed by two enzymes, Trp decarboxylase (TDC) and tryptamine hydroxylase (TH), respectively. Tyrosine decarboxylase (TYDC) is responsible for the biosynthesis of tyramine.](image-url)
was performed to identify a domain responsible for the amine-binding specificity, as well as key amino acids in pepper SHT and THT proteins.

RESULTS

Isolation and Characterization of a cDNA Clone Encoding THT

Based on the sequence of a pepper expressed sequence tag (EST; http://plant.pdrc.re.kr/ks200201/pepper.html), an EST clone (KSO1044B06) highly homologous to tobacco THT was isolated and sequenced. The pepper cDNA clone contains a 956-bp fragment with a 50-bp 5'-untranslated region, a 738-bp open reading frame, and a 168-bp 3'-untranslated region (GenBank accession no. AY819700). The open reading frame in the clone encodes a 245-amino acid protein with a predicted molecular mass of 28,221 D. A comparison of this polypeptide with that of the previously reported pepper SHT (GenBank accession no. AF329463) revealed 78% identity between the proteins. The protein has a high level of homology with THTs from other plants, including 83%, 80%, and 71% amino acid identity with tomato, potato, and tobacco THT proteins, respectively (Farmer et al., 1999; Schmidt et al., 1999; Von Roepenack-Lahaye et al., 2003). In the phylogenetic tree based on amino acid sequence of N-hydroxycinnamoyl-transferases from four plant species, pepper SHT showed a greater evolutionary distance from pepper THT than other THTs within species (Fig. 2B).

Enzymatic Properties of the Recombinant THT Enzyme

To verify that the pepper cDNA encodes a protein with THT activity, a His-tagged recombinant form of pepper THT was produced in E. coli, purified using affinity chromatography, and characterized (Fig. 3A). The enzyme kinetics of the purified His-tagged pepper THT were investigated using two cinnamoyl-CoA esters as acyl donors and a series of amines as acceptors (Fig. 3B). The reaction velocity (V_max) of the recombinant pepper THT was highest for feruloyl-CoA (67 nkat mg⁻¹), followed by p-coumaroyl-CoA (27 nkat mg⁻¹). Of the acceptor amines tested, tyramine showed the maximum V_max value (67 nkat mg⁻¹), followed by dopamine (27 nkat mg⁻¹), when feruloyl-CoA was used as the acyl donor. Tryptamine had a negligible V_max of 1 nkat mg⁻¹, and no serotonin was detected.

Figure 2. Comparison of the deduced amino acid sequences of SHT (GenBank accession no. AF329463) and THT (GenBank accession no. AY819700), and a phylogenetic tree of THTs and SHT. A, Identical amino acids are denoted as dots, and gaps are indicated by dashes. The conserved acyl-CoA binding sites are underlined (RxxxGxx and FYxxxG represent domains I and II, respectively). B, Phylogenetic analysis was performed using the PHYLODRAW program (http://pearl.cs.pusan.ac.kr/phylodraw). NtTHT1 and NtTHT represent THTs from tobacco (AJ131768 and AJ005062); LeTHT1-3, LeTHT7-8, and LeTHT7-1 are THTs from tomato (AY081905, AY081907, and AY081906); SHT denotes a THT from potato (AB061243); and CaSHT is from pepper (AF329463).

Figure 3. Affinity purification and substrate specificity analysis of the E. coli-expressed His-tagged THT protein. A, Expression of the pepper THT gene (AY819700) in E. coli. Protein samples were separated by SDS-PAGE and stained using Coomassie Blue. Lane M, Molecular standard; lane 1, total proteins in a 10-μL aliquot of bacterial cells grown without IPTG; lane 2, total proteins in a 10-μL aliquot of bacterial cells after IPTG treatment; lane 3, 20 μg of soluble proteins; lane 4, the THT protein (10 μg) purified by affinity (Ni-NTA) chromatography. B, Substrate specificity of the purified recombinant THT protein. Footnote a, Tyramine (1 mM) was used as the acyl acceptor. Footnote b, Feruloyl-CoA (250 μM) was used as the acyl donor. nd, Not detected.
conversion was detected. p-Coumaroyl-CoA had a $K_m$ value 2.5-fold less than that for feruloyl-CoA when tyramine was used as the acyl acceptor. Based on the $V_{\text{max}}/K_m$ values, both acyl donors appear to be equally converted. In contrast to the acyl donors, tyramine was the preferred substrate, with a $K_m$ of 40 $\mu$M, which is approximately 20 times less than that of dopamine, 779 $\mu$M. Other acyl acceptors, such as tryptamine or serotonin, were not accepted as THT substrates. A preference for tyramine as the acyl acceptor was clearly shown by the $V_{\text{max}}/K_m$ values, which for tyramine was 56 times higher than for dopamine. These substrate specificity data clearly show that the enzyme encoded by the pepper cDNA clone (KS01044B06) has THT enzyme activity.

**Differential Expression of SHT and THT in Pepper Tissues**

Full-length SHT and THT cDNA fragments were separated by agarose gel electrophoresis, and two identical DNA blots were hybridized independently with $^{32}$P-labeled SHT and THT cDNA clones (Fig. 4A). Under high-stringency hybridization and washing conditions, each DNA showed specific hybridization to its complementary sequence, despite the 77% identity of the SHT and THT nucleotide sequences. The differential and organ-specific expression of SHT and THT genes in mature pepper plants is shown in Figure 4B. Both the SHT and THT mRNAs were expressed constitutively in all tissues analyzed, but their expression levels in the different tissues varied. The highest amounts of THT transcripts were present in young stems, followed by roots, whereas peak levels of the SHT mRNA were observed in flowers and stems. Even though both the SHT and THT mRNAs were abundant in young stems, the THT mRNA was preferentially detectable in roots as compared to the SHT mRNA, which was present at higher levels in flowers. This result may suggest that THT and SHT genes have different biological functions in pepper plants.

**Determination of the Amine-Binding Specificity Domain**

Pepper THT and SHT are responsible for the production of the HCAAs of tyramine and serotonin, respectively. Although the kinetics of these enzymes have been well documented, no information is currently available concerning their substrate specificity or catalytic domains. THT prefers serotonin as an acyl acceptor and also has a low substrate specificity affinity toward tyramine, but we found that THT only uses tyramine as an acyl acceptor, with no affinity for serotonin. Both the high amino acid identity and the distinct substrate specificities of the two proteins prompted us to generate chimeric proteins to identify domains that might contribute to the amine-binding specificities for tyramine or serotonin. Chimeric genes were generated by swapping corresponding domains between the SHT and THT genes, after which the chimeric genes were expressed in bacteria as described in “Materials and Methods.” The recombinant chimeric proteins, each fused with a hexa-His tag, were affinity purified (Fig. 5A) and assayed for SHT and THT activity (Fig. 5B). Five chimeric constructs were generated and assayed. The specific activities of SHT, which has bifunctional enzyme activity, were 6.5 and 17 nkat mg$^{-1}$ protein for SHT and THT, respectively. In contrast, THT exhibited only THT-specific activity, with 67 nkat mg$^{-1}$ protein. Chimera 1 (CH1) showed only THT activity, with a specific activity similar to the original THT. This result suggests that the carboxy-terminal halves of the two proteins contribute to the specificity of the amine-binding reaction. CH5 is the converse construct of CH1, containing the THT amino terminus and the SHT carboxy terminus, and the specificity would be expected to be that of SHT. A comparison of CH1 and CH2 provides evidence that the amine-binding specificity resides within a domain of approximately 37 amino acids corresponding to amino acids 129 to 165 of the SHT sequence. This interpretation was further confirmed by the results from constructs CH3 and CH4. CH3 represents a substitution of amino acids 125 to 160 from THT with the corresponding amino acids of SHT and exhibits SHT activity. CH4 is a substitution of amino acids 129 to 165 of SHT with the corresponding THT sequence and results in THT-specific activity. This generation and characterization of a series of chimeric genes has demonstrated unequivocally that the amine-binding specificity domain resides within amino acid residues 129 to 165 of SHT and the counterpart residues 125 to 160 of THT.

**Catalytic Specificities of the Recombinant Chimeric Proteins**

To examine more closely the mechanistic roles of the amine substrate specificity domains in these chimeric
proteins, their kinetic constants were determined. As shown in Table I, the kinetic parameters of the different proteins spanned a remarkably wide range. The affinities for tyramine and serotonin varied 416- and 7- fold, respectively. Wild-type SHT and THT exhibited distinct substrate specificities, with the former having a higher specificity for serotonin and an approximately 7-fold decrease in the $V_{\text{max}}$ for tyramine, as shown with the SHT-type chimeras (CH2, CH3, and CH5), but that the THT specificity domain tended to lower the $V_{\text{max}}$ values for serotonin (as seen with CH1 and CH4). Unlike the $V_{\text{max}}$ values, all of the chimeras had high $K_m$ values for both tyramine and serotonin.

**Catalytic Specificities of Point-Mutated Chimeric CH3 Proteins**

Through the generation and characterization of a series of chimeric genes, we identified the amine substrate specificity domain of the SHT and THT proteins (Fig. 6A). An alignment of the amino acid sequences of these domains is shown in Figure 6B. The amine substrate specificity domains consist of 33 and 32 amino acids in SHT and THT, respectively, differing by seven amino acids. Two (Tyr or Phe at 149 and Phe or Tyr at 151, based on SHT numbering) of these seven amino acids were substituted by similar amino acids, and two others (Arg at 130 and Thr at 137) were variable between the pepper SHT and the various THTs. The amino acid residues that are distinctly different between SHT and THT are Ser, Leu, and Gly at 153, 156, and 158, respectively, of the SHT sequence. To verify the roles of these amino acid residues, we first employed a CH3 chimeric gene as a template for point mutation. The point-mutated CH3 chimeric genes were expressed in *E. coli* and affinity purified (Fig. 6C). To characterize the point-mutated CH3s, we assayed the $N$-hydroxycinnamoyltransferase activities of the mutated CH3s using tyramine and serotonin as acyl acceptors. All three of the mutated CH3 genes containing mutations within the amine substrate specificity domain (S153N, L156C, and G158Y) displayed lower SHT activity, whereas the THT activity was increased by 342%. Similarly, L156C, in which the Tyr at 156 of SHT was replaced with the Cys of THT, exhibited a 41% lower SHT activity, whereas the THT activity increased by 320%. These results suggest that Ser-153, Leu-156, and Gly-158 have important roles in regulating the amine substrate specificity of SHT. Next, we investigated the kinetic constants of the mutated CH3 proteins to determine whether their specific activities are associated with more profound effects on the substrate specificity kinetics. As shown in Table II, the CH3
chimera displayed bifunctional substrate specificities with a \( V_{\text{max}}/K_m \) value for serotonin that was higher than that for tyramine. In agreement with the specific activity results obtained from the three mutated CH3 proteins, all three, in which residues present in the amine substrate specificity domain were mutated, exhibited different \( V_{\text{max}}/K_m \) values with either tyramine or serotonin as substrate because of variations in the \( V_{\text{max}} \) and \( K_m \) values, as compared to those of the unmutated CH3. All three of the mutated CH3 proteins exhibited decreased \( V_{\text{max}}/K_m \) values for serotonin, ranging from 18% to 60% of that of the original CH3, whereas the catalytic efficiency with tyramine as the substrate, as reflected by the \( V_{\text{max}}/K_m \) toward tyramine by an average of 2-fold. It is particularly notable that the G158Y mutation resulted in a 3-fold increase in the \( K_m \) for serotonin, whereas the \( V_{\text{max}} \) decreased by 2-fold, leading to a 5-fold decrease in

<table>
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<th>Enzyme</th>
<th>( K_m ) (( \mu \text{M} ))</th>
<th>( V_{\text{max}} ) (nkat mg(^{-1}))</th>
<th>( V_{\text{max}}/K_m )</th>
<th>( K_m ) (( \mu \text{M} ))</th>
<th>( V_{\text{max}} ) (nkat mg(^{-1}))</th>
<th>( V_{\text{max}}/K_m )</th>
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<td>0.0146</td>
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<td>ND</td>
<td>ND</td>
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<tr>
<td>CH1</td>
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<td>ND</td>
<td>ND</td>
<td>ND</td>
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<td>0.00115</td>
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**Figure 6.** Comparison of amino acid sequences in the amine substrate specificity domain and specific activities of mutated CH3 proteins. A, Schematic diagram of the amine substrate specificity domain. B, Comparison of the amino acid sequences of the N-hydroxycinnamoyltransferases. The sequence of pepper SHT (AF329463) was aligned with the THT sequences from pepper (AY819700), tobacco (AJ131768), potato (AB061243), and tomato (AY081907). Identical amino acids are denoted as dots, and gaps are indicated by dashes. C, Expression of the mutated CH3 genes in *E. coli* and affinity purification of His-tagged mutated CH3 constructs. Lane M, Molecular standard; lane 1, total proteins in 10 \( \mu \text{L} \) of bacterial cells of the CH3-S153N strain grown without IPTG; lane 2, total proteins in 10 \( \mu \text{L} \) of bacterial cells of the CH3-S153N strain after IPTG treatment; lane 3, 20 \( \mu \text{g} \) of soluble proteins from the CH3-S153N strain; right lanes (S153N, L156C, and G158Y), mutated CH3 proteins (10 \( \mu \text{g} \)) purified by affinity (Ni-NTA) chromatography. D, Constructs of point-mutated CH3 chimeras and measurements of their specific activities. THT-SLG is a THT protein containing three mutated residues in which Asn-149, Cys-152, and Tyr-154 were replaced with the corresponding Ser-153, Leu-156, and Gly-158 of SHT, respectively. Enzyme assays were performed as described in Figure 5B.
the $V_{\text{max}}/K_m$ values. In contrast to the results for serotonin, G158Y exhibited $K_m$ and $V_{\text{max}}$ values for tyramine that were increased by 1.5- and 3.4-fold, respectively. This resulted in a greater than 2-fold increase in the $V_{\text{max}}/K_m$ with tyramine as substrate. Similar to the G158Y mutant, mutations at S153N and L156C resulted in an increase in the $V_{\text{max}}/K_m$ with tyramine and a decrease in the $V_{\text{max}}/K_m$ with serotonin. These results indicate that these mutations at the three amino acids are sufficient to cause significant changes in the substrate specificity from serotonin to tyramine. However, none of these mutations was able to abolish the SHT activity of the bifunctional CH3 chimera. This result was further confirmed by constructing THT-SLG, in which Asn-149, Cys-152, and Tyr-154 of THT were replaced with the Ser-153, Leu-156, and Gly-158 of SHT. The NCY/SLG-mutated THT exhibited a significant decrease in the specific activity as compared to that of wild-type THT (Fig. 6D). The reduced specific activity was accompanied by a significant decrease in the substrate affinity and catalytic efficiency, with an approximately 425-fold increase in the $K_m$ and a 7-fold decrease in the $V_{\text{max}}$. The concerted action of the three mutated residues (THT-SLG) resulted in a substantial reduction in the THT catalytic efficiency rather than a gain in the SHT activity (Table II).

### Catalytic Specificities of Point-Mutated SHT Proteins

To confirm that the three amino acid residues mentioned above also play a significant role in regulating the catalytic activity of SHT protein, we also point mutated and affinity purified SHT proteins, as was done with the chimeric CH3 proteins. Furthermore, we point mutated two more amino acids, Tyr-149 and Phe-151, which were substituted by similar amino acids between SHT and THT. With the single point-mutated SHT proteins purified as above (Fig. 7A), the SHT- and THT-specific activities showed significant dissimilarities compared to those of the wild-type SHT protein (Fig. 7B). For example, the replacement of either Ser with Asn at 153 (SHT-S153N) or Leu with Cys at 156 (SHT-L156C) had an additive effect on the specific activities of THT by 2.8- and 2.1-fold, respectively. However, when Gly was replaced with Tyr at 158 (SHT-G158Y), the enzyme activity of THT decreased about 2.9-fold, whereas the SHT activity increased by about 4-fold, suggesting a crucial role of Gly for THT activity in the SHT enzyme. As for the three-point-mutated SHT (SHT-NCY), the specific activity of SHT increased 2-fold, but THT activity decreased about 2.3-fold. As compared to SHT-G158Y, SHT-NCY had 25% higher specific activity for THT, suggesting that these two amino acids (i.e. Ser-153 and Leu-156) are necessary to sustain a high rate of THT activity. Other mutant constructs, such as SHT-Y149F, SHT-F151Y, and SHT-FY, showed low THT and SHT specific activities relative to wild-type SHT, except the SHT-Y149F with a 1.2-fold increase of SHT activity.

The pivotal role of these amino acids was clearly evident when judged using kinetic analyses (Table II). For example, the catalytic efficiency ($V_{\text{max}}/K_m$) with tyramine increased 2.8-fold in SHT-S153N as compared to wild-type SHT. The $V_{\text{max}}/K_m$ value of SHT-L156C increased in a manner similar to that of SHT-S153N, implicating Ser-153 and Leu-156 in THT catalysis, rather than SHT catalysis. Additionally, the SHT-L156C exhibited the decrease in $K_m$ for serotonin by 1.7-fold, resulting in a new type of bifunctional SHT enzyme with a higher catalytic efficiency toward both tyramine and serotonin substrates relative to wild-type SHT. Unlike SHT-S153N and SHT-L156C, SHT-G158Y resulted in a dramatic decrease in $V_{\text{max}}/K_m$ with tyramine because of a 13-fold increase in $K_m$ and a 3-fold decrease in $V_{\text{max}}$ as compared to wild-type SHT. This is in contrast with the $V_{\text{max}}$ value with

### Table II. Kinetic constants of mutated CH3 and SHT proteins with tyramine and serotonin as amine substrates

Feruloyl-CoA (250 μM) was used as the acyl donor. ND, Not detected.

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<th>Enzyme</th>
<th>Tyramine</th>
<th>Serotonin</th>
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<td></td>
<td>$K_m$ μM</td>
<td>$V_{\text{max}}$ nkat mg$^{-1}$</td>
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<td>CH3</td>
<td>16,658</td>
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<tr>
<td>CH3-S153N</td>
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</tbody>
</table>
serotonin, which increased 4-fold compared to wild-type SHT. Again, these data suggest that Gly-158 is essential for catalysis and substrate binding, especially with tryptamine. SHT-NCY, which contained three amino acid substitutions (S153N, L156C, G158Y), showed a similar catalytic efficiency as compared to SHT-G158Y. Contrary to the specific activity data, SHT-Y149F transformed SHT enzymes into the THT type by reversing the substrate affinity toward serotonin and tryptamine. The $K_m$ for serotonin in the SHT-Y149F was increased by 21-fold, but the $K_m$ for tryptamine was reduced by 7-fold, with a marginal change of corresponding $V_{max}$ values resulting in a higher catalytic efficiency for tryptamine ($V_{max}/K_m = 0.0968$) than for serotonin ($V_{max}/K_m = 0.0049$). This phenomenon is corroborated by the SHT-FY double mutant in which the catalytic efficiency for tyramine is 4-fold higher than for serotonin. SHT-F151Y plays a role in modulating substrate affinity, but its affinity to serotonin is still higher ($K_m = 450 \mu M$) than tyramine ($K_m = 783 \mu M$). The determining role of Tyr-149 in wild-type SHT for regulating a substrate preference between serotonin and tyramine was further confirmed on the kinetics and substrate specificity (Fig. 7C). The $K_m$ values in the SHT-Y149F for two acyl donors, $p$-coumaroyl-CoA and feruloyl-CoA, were 25 and 5 $\mu M$, respectively, which are comparable to those measured in wild-type SHT (Jang et al., 2004). This indicates that the mutation in Tyr-149 into Phe-149 does not alter acyl donor substrate binding or catalysis. The $V_{max}$ for various acyl acceptors differed within the range of 160 to 2,213 $\mu M$ in the SHT-Y149F with a higher affinity for tyramine ($K_m = 160 \mu M$) than for serotonin ($K_m = 1,513 \mu M$) when feruloyl-CoA was used as the acyl donor. Thus, $K_m$ for tyramine is 9-fold lower than for serotonin, suggesting that the SHT-Y149F mutant behaves like a THT enzyme. In contrast to wild-type THT, the SHT-Y149F mutant has an additional ability to accept serotonin and tryptamine as acyl acceptors. Collectively, this result clearly reveals that Tyr-149 is a critical amino acid residue for enabling wild-type SHT to accept serotonin with a high affinity.

Conversion from THT to SHT by Mutating Phe-145 to Tyr in THT

Given the key role played by Tyr-149 in SHT in regulating the serotonin specificity, it is of great interest to examine whether the mutant THT enzyme, in which a Phe residue (F145Y) is mutated to a Tyr residue, gains SHT activity by accepting serotonin as the acyl acceptor. As shown in Figure 8, THT-F145Y is capable of utilizing serotonin as a substrate, showing SHT activity in addition to THT activity. Hence, the replacement of Phe-145 with Tyr in THT contributes pivotally to the change in accommodating serotonin as a substrate, resulting in a novel bifunctional THT enzyme like SHT. The kinetic constants of THT-F145Y differed from those of wild-type THT (Fig. 8, B and C). THT-F145Y possessed approximately 3-fold less THT-specific activity than wild-type THT, but acquired a novel SHT activity of 8 nkat mg$^{-1}$ protein. Interestingly, THT-F145Y had increased affinity for tyramine ($K_m = 17 \mu M$), and its $V_{max}$ value decreased by approximately 3-fold relative to that of wild-type THT. In addition, THT-F145Y had a $K_m$ for serotonin of 20,980 $\mu M$ and a $V_{max}$ of 8.2 nkat mg$^{-1}$ V. The double mutant THT-FY145,147YF showed kinetic values similar to those of THT-F145Y, indicating that Phe-145 in THT is primarily involved in regulating amine-binding specificity.
DISCUSSION

It has been well documented that THT enzymes have a broad range of substrate specificities and are encoded by a small gene family (Farmer et al., 1999; Schmidt et al., 1999). Tobacco and potato contain at least three THT isogenes, as shown by genomic Southern analyses. It would be intriguing to know whether all of the isogenes encode functional THT enzymes with HCAA synthesis activity and whether they display similar substrate specificities. With this aim, Von Roepenack-Lahaye et al. (2003) isolated and characterized four THT isogenes from tomato. The deduced amino acid sequences exhibited 93% similarity. However, only three of the isogenes encoded functional THT enzymes, each with the highest affinity toward tyramine as the acyl acceptor. Interestingly, the second best acyl acceptor varied among the three THT enzymes, suggesting that the THT isogenes encode proteins with different substrate specificities and physiological roles. The presence of a small gene family with the distinct characteristics of the THT isogenes was demonstrated in pepper. The two highly similar THT isogenes identified have been demonstrated to encode two different enzymes, THT and SHT. In addition to its presence in the Solanaceae, THT activity has been observed in other plant families, such as the Papaveraceae (Yu and Facchini, 1999) and Gramineae (Ishihara et al., 2000; Jang et al., 2004), but no gene structure data have been reported as yet.

SHT and THT belong to the GCN5-related family of structurally similar N-acetyltransferases, which includes streptothricin acetyltransferase, gentamicin 3'-acyltransferase, aminoglycoside 6'-N-acetyltransferase, spermidine/spermine N-acetyltransferase, and serotonin N-acetyltransferase, although SHT and THT do not share extensive homology with these proteins (Lu et al., 1996). Nevertheless, all of these enzymes have molecular masses of around 20 to 25 kD and contain two conserved domains responsible for acetyl-CoA binding and enzyme activity. The domains I and II of the N-acetyltransferases, which have the amino acid sequences RGFGIG and FYXRXG, are well conserved, as compared to the RKLGMG and FYXXG sequences of SHT and the THTs, respectively. Interestingly, the distance between domains I and II and their locations in the C-terminal portion of the N-acetyltransferase proteins are also well conserved. The pivotal roles of these domains were further confirmed by the determination of the three-dimensional structure of serotonin N-acetyltransferase (Hickman et al., 1999), which showed that domain I is involved in acetyl-CoA binding and domain II is associated with substrate binding. Furthermore, this study indicates that Tyr-168 may play a role in the reprotonation of the thiolate leaving group as a general acid. In contrast, our study of chimeras of SHT and THT showed that domain II is not implicated in amine substrate binding but contains the common domain for enzyme activity. The domain for amine substrate specificity is located within amino acids 129 to 165, a sequence that is upstream of domain I by only 10 amino acids. The amino acid sequences of the amine substrate domains of pepper SHT and pepper THT have 76% identity and 82% similarity. However, whether the entire stretch of amino acid residues is necessary for determination of the substrate by the SHT enzyme, controlling the accessibility of the bulky substrate serotonin versus the smaller substrate tyramine, is unknown. To clarify this issue, we generated several mutated SHT proteins to determine whether a single point mutation could affect the substrate specificity of SHT. Of the five mutants in wild-type SHT, mutation of Tyr-149 to Phe (SHT-Y149F) dramatically reverses the substrate affinity from serotonin to tyramine, indicating that this residue plays a key role in serotonin substrate binding and catalysis. The essential role of Tyr-149 in SHT seems to be different from that of Tyr-168 as a general acid in the serotonin N-acetyltransferase, whose mutation of Tyr-168 to Phe significantly reduces its catalysis (Hickman et al., 1999). In contrast, mutation of Tyr-149 to Phe in the SHT did not affect either for serotonin or tyramine, suggesting that Tyr-149 may not function as a general acid for amine substrates. It is therefore probable that Tyr-149 could serve to accurately place a
SEROTONIN SUBSTRATE WITH AN UNKNOWN TYR RESIDUE PLACED AS A GENERAL ACID IN THE ACTIVE POCKET.

OTHER MUTANT CONSTRUCTS WERE ALSO CAPABLE OF MODULATING THE SPECIFIC ACTIVITY RATIO IN KEEPING WITH VARYING $K_a$ AND $V_{\text{max}}$, BUT NONE OF THE MUTATIONS, INCLUDING Y149F, COMPLETELY ABOLISHED THE SEROTONIN OR TYRAMINE SUBSTRATE SPECIFICITY IN SHT. THIS, IT IS HIGHLY LIKELY THAT THE MUTANT THT ENZYMES, IN WHICH A PHE RESIDUE (F145Y) IS MUTATED BY A TYR, WILL BE A BIFUNCTIONAL ENZYME ACCEPTING BOTH SEROTONIN AND TYRAMINE AS THE ACYL ACCEPTORS AS SHOWN IN THE CHIMERIC CH3. THIS HYPOTHESIS WAS FURTHER TESTED AND CONFIRMED BY CONSTRUCTING THE THT-F145Y IN WHICH THE MUTANT THT-F145Y TURNED OUT A BIFUNCTIONAL ENZYME CATALYZING SEROTONIN AND TYRAMINE AS THE ACYL ACCEPTORS.

IN ADDITION, IT IS WORTH NOTING THAT SHT-L156C HAD 1.6-FOHIGER CATALYTIC EFFICIENCIES WITH BOTH SEROTONIN AND TYRAMINE, RESPECTIVELY, AS COMPARED TO WILD-TYPE SHT. THIS SUGGESTS THAT IT IS POSSIBLE TO GENERATE EITHER THT WITH GREATER CATALYTIC EFFICIENCY FOR A TYRAMINE SUBSTRATE ONLY OR A BIFUNCTIONAL ENZYME WITH A HIGHER SUBSTRATE AFFINITY FOR THE SPECIFIC AMINE SUBSTRATES (I.E., SEROTONIN AND SEROTONIN) THROUGH A SERIES OF SITE-DIRECTED MUTAGENESIS WITHIN VARIOUS N-HYDROXYCINNAMOYLTRANSFERASES FOUND IN NATURE.

MATERIALS AND METHODS

ISOLATION AND SEQUENCE IDENTIFICATION OF A THT CLONE


BACTERIAL EXPRESSION AND ENZYME PURIFICATION


CHIMERIC GENES

CONSTRUCTION AND SITE-DIRECTED MUTAGENESIS OF CH1


CONSTRUCTION AND SITE-DIRECTED MUTAGENESIS OF CH3 CHIMERIC GENES

SITE-DIRECTED MUTAGENESIS OF THE CH3 CHIMERIC GENE WAS PERFORMED USING IN VITRO DNA SYNTHESIS AND PCR WITH MUTAGENIC PRIMERS. FIRST, A POINT-MUTATED AMINE-BINDING DOMAIN REPRESENTING AMINO ACIDS 125 TO 161 IN THE CH3 CHIMERIC GENE WAS SYNTHESIZED IN VITRO WITH THE MUTAGENIC PRIMERS. THE FORWARD PRIMER WAS 66 BP IN LENGTH AND HAD THE SEQUENCE 5'-d(GAAGATGTTTCTCATCAGCATGCGAATTCC)-3' AS THE REVERSE PRIMER (THE EcoRI SITE IS UNDERLINED), AND HARBORED POINT-MUTATED NUCLEOTIDES (UNDERLINED), AS FOLLOWS: 5'-d(GAAGATGTTTCTCATCAGCATGCGAATTCC)-3', THE 5' CDNA (AGT) WAS CHANGED TO AN ASN.
Construction and Site-Directed Mutagenesis of SHT Genes

Sited-directed mutagenesis of the SHT gene was performed as follows. SHT-S153N was created by replacing the EcoRI-BamHI fragment of CH4 with the corresponding pET28b-CH3 fragment. Similarly, SHT-L156C was synthesized from the corresponding sites of the CH4-S153N fragment. Both the forward primer was 60 bp long and had the sequence 5'-GGCGCCCGCTATGAAAACATCACGCTT-3'. The mutated nucleotides were shown in bold. The PCR products were digested with EcoRI and BamHI, gel purified, and fused into Chloroplast SK+ between the two restriction sites. Next, the EcoRI and BamHI fragments of the pET28b-CH3 plasmid were replaced with the point-mutated EcoRI and BamHI fragments, resulting in pET28(b)-CH3-S153N, -L156C, and -G138Y.

LITERATURE CITED


Sequencing data from this article can be found in the GenBank/EMBL databases under accession number AY819700.

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