Biochemistry and molecular biology in caffeine biosynthesis ---

Molecular cloning and gene expression of caffeine synthase.

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Summary

Purine alkaloids such as caffeine (1,3,7-trimethylxanthine) is one of the famous second metabolites in tea (Camellia sinensis) and coffee (Coffea arabica). The young flush shoots in tea contain ca.3% caffeine on dry weight basis. The available data support the operation of a xanthosine → 7-methylxanthosine → 7-methylxanthine → theobromine → caffeine pathway as the major route to caffeine in tea leaves. The two final steps of the pathway, in which two methyl groups are added successively to 7-methylxanthine to produce theobromine and then, caffeine, are catalysed by caffeine synthase (CS), a bifunctional enzyme comprising two S-adenosylmethionine-dependent N-methyltransferase activities. It has been hard to purify and caffeine synthase and other enzymes of this pathway because they are extremely labile, but we have succeeded in isolating CS. We used the RACE technique with degenerate gene-specific primers on the amino-terminal sequence of CS to obtain cDNA, termed TCS1. The cloning of the CS gene is an important advance towards the development of transgenic caffeine-deficient tea plants through antisense messenger RNA technology.

Key words
Caffeine, Caffeine synthase, N-methyltransferase, S-adenosylmethionine, Theobromine

Introduction

Caffeine is the best known purine alkaloid. Although caffeine itself was discovered in coffee and tea in the early 1820s, extensive metabolis studies of purine alkaloids in leaves of tea and coffee have elucidated the caffeine biosynthetic pathway in some detail [1-3]. The available data support the operation of a xanthosine → 7-methylxanthosine → 7-methylxanthine → theobromine → caffeine pathway as the major route to caffeine (Fig.1). The pathway contains three S-adenosylmethionine (SAM)-dependent methylation steps, indicating that N-methyltransferase paly an important role. However, little is known about the properties of N-methyltransferases which participate in the caffeine biosynthetic pathway because the activity of the cell-free preparations is extremely labile.

In the present study, N-methyltransferase, referred to caffeine synthase (CS), which catalayze the conversion of 7-methylxanthine to caffeine via theobromine was purified to apparent electrophoretic homogeneity. The molecular cloning based on N-terminal sequence of CS was performed.
Materials and Methods

*Plant material* - The young and most recently emerged developing leaves from flush shoots of tea plants growing at the experimental farm of the National Research Institute of Vegetables, Ornamental Plants, and Tea (Makurazaki, Kagoshima, Japan) were collected.

*Extraction and Purification of CS* - CS was extracted from frozen leaves (100 g fresh weight) with 1200 ml of 50 mM sodium phosphate buffer, pH 7.3, containing 5 mM 2-mercaptoethanol, 5 mM Na2-EDTA, 5 % (v/v) glycerol, 1 mg of aprotinin, 2.5 % (w/v) insoluble polyvinylpolypyrrolidone, and 0.5 % (w/v) sodium ascorbate. CS was purified from the cell-free extract as the following steps; ammonium sulfate precipitation, hydroxyapatite chromatography, anion-exchange chromatography (Shodex IEC QA-824), affinity chromatography (adenosine-agarose), and gel filtration chromatography (Superdex 200). The purification procedure was described in ref. 4.

*Molecular cloning of CS* - The N-terminal amino acid sequence of CS was used to design for PCR amplification of CS sequence fragments from cDNA. Total cellular RNAs from tea tissues were extracted by the selective precipitation method with 8 M urea in 3 M LiCl solution. Tea leaf polyadenylated RNA (5 mg) purified with an oligo(dT)-cellulose column (Pharmacia) was denatured by incubation at 65°C for 10 min. cDNA was synthesized by a single strand cDNA synthesis kit (Pharmacia). The N-terminal amino acid sequence of CS obtained from previous studies [4] was used to design for PCR amplification of CS sequence fragments from cDNA. First strand cDNAs were synthesized using First-strand cDNA Synthesis Kit (Pharmacia). PCR was conducted in a PTC-2000 thermal cycler (MJ Research) for 30 cycles (1 min at 95°C, 1 min at 45°C, 2 min at 72°C) with the first strand cDNAs and the N-terminal specific primer (5'-TTYATGAA YMGGIGGARG-3'). The resulting PCR products were subcloned into the pT7Blue vector (Novagen). The nucleotide sequences of the 5' regions of TCS clone was obtained by 5'-RACE (Rapid Amplification of cDNAs ends) with the 5' Full RACE set (TaKaRa) according to the manufacturer's protocol. The first cDNA strand was primed with the two different types of the dephosphorylated oligonucleotides, (5'-CGGCCATGGAAAGACCCCGG-3') or (5'-CAGCAATGGCCATAGCTAATAG-3'). After the self-ligation of the two types of cDNA, the obtained concatemers were used for a nested PCR amplification, individually. The primer sets for 5'-RACE were as follows: TCS-2R (5'-CCGCTGCTTAAGACCTTGAAG-3') and TCS-3F (5'-GCCAAACACTGGAACCTCAGG-3') or TCS-1R (5'-CATTGAGGCACCTGTCGTG-3') and TCS-4F (5'-GGCCTGTCGTAGTTAATTG-3'). 30 cycles of PCR was conducted for 30 cycles set to 94°C for 30 sec, 50°C for 30 sec, 72°C for 1.5 min (1st PCR) and 94°C for 30 sec, 52°C for 30 sec, 72°C for 1.5 min (2nd PCR). Desired amplification products were isolated by polyacrylamide gel purification, cloned into pT7 blue vector and sequenced.

*Expression of TCSI in E.coli* - The full-length coding region for CS protein was ligated into pET23d plasmids and the resultant expression vector introduced into E. coli (BL21). Recombinant CS protein was extracted by sonication of the transformed cells in 50 mM Tris-HCl (pH7.5) containing 1 mM EDTA-Na2 and 0.1 M NaCl. The substrate specificity of the recombinant CS was determined according to ref. 4.
Northern blot analysis - 5 μg of total RNA from each organ of tea leaves was electrophoresed on 1.2% agarose gels, and then blotted to the membrane as described previously [5].

Results and Discussion

Purification of CS - The results of a typical purification table were summarized in Table 1. The recovery of the CS activity was 3.6%, accompanied by a 523-fold increase in specific activity at 5700 pkat mg⁻¹ protein. The key step was affinity chromatography on adenosine-agarose. This method was previously used with success to purify an S-methyltransferase of plant origin [6]. The native enzyme was monomeric with an apparent molecular mass of 61 kD as estimated by gel-filtration chromatography and 41 kD as analyzed by SDS-PAGE. The substrate specificity of the final enzyme preparation was summarized in Table 2. The purified CS exhibited 3- and 1-N-methyltransferase activity with a broad substrate specificity, showing high activity toward paraxanthine, 7-methylxanthine and theobromine and low activity with 3-methylxanthine and 1-methylxanthine. However, the enzyme had no 7-N-methyltransferase activity toward xanthosine and xanthosinemonophosphate. The broad substrate specificity of the purified CS is very similar to that of crude tea-leaf extract [7, 8], suggesting that CS is a major N-methyltransferase in tea leaves.

Molecular cloning of TCS1 - The isolated cDNA by RACE technique with degenerate gene-specific primers based on the amino-terminal sequence of CS, termed TCS1 (accession no. AB031280), consists of 1,438 bp and encodes a protein 369 amino acids. The deduced amino acid sequence of TCS1 shares almost only 40% identity with those of CCS genes, which encode specific 3-N-methyltransferase in coffee [9]. Moreover, it shares a small amount of sequence similarity with any other N-, S- and O-methyltransferases from plants and microorganisms, but considerably more with the salicylic
acid O-methyltransferase (41.2 %) [10]. To determine whether cDNA encoded an active caffeine synthase enzyme, the recombinant TCS1 was expressed in E. coli. The substrate specificity of the lysates of the bacterial cells containing the recombinant TCS1 was very similar to that of the native enzyme (Table 2). The obtained data indicates that TCS1 encodes caffeine synthase.

Table 2: Substrate specificity of recombinant and native caffeine synthase

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Methylated product</th>
<th>N-methylation position</th>
<th>Recombinant CS</th>
<th>Native CS</th>
</tr>
</thead>
<tbody>
<tr>
<td>7-Methylxanthine</td>
<td>Theobromine</td>
<td>3</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>3-Methylxanthine</td>
<td>Theophylline</td>
<td>1</td>
<td>1.0</td>
<td>17.6</td>
</tr>
<tr>
<td>1-Methylxanthine</td>
<td>Theophylline</td>
<td>3</td>
<td>12.3</td>
<td>4.2</td>
</tr>
<tr>
<td>Theobromine</td>
<td>Caffeine</td>
<td>1</td>
<td>18.5</td>
<td>26.8</td>
</tr>
<tr>
<td>Theophylline</td>
<td>Caffeine</td>
<td>7</td>
<td>&lt;0.1</td>
<td>&lt;0.1</td>
</tr>
<tr>
<td>Paraxanthine</td>
<td>Caffeine</td>
<td>3</td>
<td>230</td>
<td>210</td>
</tr>
<tr>
<td>Xanthosine</td>
<td>7-Methylxanthosine</td>
<td>7</td>
<td>not detected</td>
<td>not detected</td>
</tr>
</tbody>
</table>

Caffeine synthase activity of the recombinant enzyme with 7-methylxanthine (100%) was 5.4 pkat mg⁻¹ protein; values represent the average of duplicate samples.

Expression of TCS1 in tea leaves - We carried out Northern blot analysis of total RNAs from tea leaves using a 527 bp cDNA fragment of the central region of TCS1 as a probe. mRNA signal for TCS1 were detected in developing leaves at high levels, and reached to the low level in old leaves (Fig. 2). These expression patterns were similar to that of the caffeine synthase activity in tea leaves [11].

Further prospective - The cloning of the caffeine synthase gene is an important advance towards the development of transgenic caffeine-deficient tea and coffee plants through antisense mRNA technology or by gene silencing. We are trying to establish the transgenic tea plants in which gene expression of CS is blocking by antisense-mRNA.

References