Note

Comparative Characterization of the oah2 Gene Homologous to the oah1 of Thermus thermophilus HB8

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The oah2 gene homologous to the oah1 of Thermus thermophilus HB8 was cloned and sequenced. It comprised 1,236 bp encoding a protein of 412 amino acid residues and was overexpressed. The gene product, also having O-acetyl-L-homoserine sulfhydrylase (EC 4.2.99.10) activity, was purified to homogeneity and characterized comparatively with the oah1 product. The two proteins shared many characteristics.

Key words: O-acetylhomoserine sulfhydrylase; Thermus thermophilus HB8; oah1; oah2

Direct synthesis of homocysteine by O-acetyl-L-homoserine (OAH) sulfhydrylase (SHase) (EC 4.2.99.10) has been described by Kosuge et al.1) to be dominant in an extremely thermophilic bacterium, Thermus thermophilus HB27, on the basis that deficiency in a gene homologous to the Saccharomyces MET17 gene2) results in a homocysteine auxotroph. We considered that T. thermophilus HB8 can synthesize homocysteine through transulfuration, after observing the presence of cystathionine (CTT) in the reaction mixture of CTT-γ-synthesis with a cell-extract employed as enzyme. The behaviors of enzymes related to CTT metabolism in cells fed with various sulfur compounds also support this conclusion.3) But recently we have learned by carrying out direct determination of CTT-γ-synthese activity (rates of cysteine consumption and CTT production) that the activity in the cell-extract is so low that more evidence is needed for it to be concluded that the transsulfuration functions physiologically. We have also discussed the possible occurrence of two proteins having OAH SHase activity.3) We considered that an analysis of their characteristics might yield information concerning sulfur incorporation in this organism. Cloning of one gene (oah1) homologous to Escherichia coli metB (encoding CTT-γ-synthese) from T. thermophilus HB8, overexpression of it, and purification and characterization of the gene product (OAH1 protein) have been performed.4) The purified preparation was found to be almost inactive in catalyzing CTT-γ-synthesis, although it shows strong OAH SHase activity. The catalytic property of the protein is not contradictory on the basis of a result of the nucleotide sequence analysis viz., that oah1 shows an identity of deduced amino acid sequence of 30% to the metB gene, although it shows much higher identities (42–56%) to genes encoding OAH SHases of several organisms.5) Another gene (oah2), also homologous to E. coli metB (with 32% identity) and to the oah1 (with 43% identity), has been found in the genome of the same organism by Kuramitsu et al. (personal communication). Although it is not highly probable that the oah2 gene encodes CTT-γ-synthese in view of its insufficient identity to the metB gene, we have been interested in investigating the reason for the presence of two such genes in the cell and their roles. In order to obtain a clue to these subjects, we attempted to characterize the oah2 gene and its product. In this report, we describe cloning of the oah2 gene from T. thermophilus HB8, overexpression of it in E. coli cells, and purification and comparative characterization of the gene product (OAH2 protein) with OAH1 protein.

Cloning and overexpression of the gene were carried out substantially according to previous work on oah1.4) Using information provided by the T. thermophilus HB8 genome project, we synthesized two primers for amplification of the DNA fragment containing the oah2 gene by polymerase chain reaction (PCR): 5'-GATTCA-TATGGAATACACCCTCGCGGT-3' and 5'-TG-TAGGATCCCTAGACC0CCCTGCGGCCT-3'. Underlining indicates the NdeI site and the BamHI site in that order. The amplified gene fragment was cloned into pT7Blue to give a plasmid pTOAH2. The nucleotide sequence of the amplified fragment (ca. 1,200 bp) was confirmed by DNA sequencing. The fragment containing the oah2 gene was ligated into the NdeI–BamHI region of pET11a (pEOAH2). Transformation of an E. coli strain, culture to overexpress the gene, purification of the gene product (from 50.4 g of transformed cells) were done essentially in the same way as

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Abbreviations: CTT, L-(+)-cystathionine; K–P, potassium phosphate; OAH, O-acetyl-L-homoserine; PLP, pyridoxal 5'-phosphate; SHase, sulfhydrylase
previously described.4) The SHase reactions with O-substituted amino acids, their activity determination, and other conditions were the same as described previously.4) One unit (U) of the enzyme was defined as the amount catalyzing the production of 1 μmol of homocysteine or cysteine per min. The CTT γ-synthese reaction was carried out at 50°C in a mixture (1 ml) comprising 20 mM potassium phosphate (K–P) buffer (pH 7.8), 1 mM EDTA, 1 mM DTT, 0.2 mM PLP, 10 mM OAH, 1 mM L-cysteine, and 45 μg of the protein. CTT γ-synthese activity was determined by observing change in the cysteine concentration according to the method of Gaitonde.5) Gel filtration was carried out on a TSKgel G3000SW column (0.75 × 30 cm; Tosoh, Tokyo, Japan) equilibrated with 50 mM K–P buffer (pH 7.8) containing 0.1 M NaCl for MW estimation, with standard proteins. Protein concentration was determined using bovine serum albumin as a standard according to the method of Bradford.5) Sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out according to the method of Weber and Osborn.7) Amino acid sequence alignment was carried out using Clustal W.8) The sequence data have been submitted to the DDBJ, EMBL, and GenBank databases under accession no. AB094564.

A PCR product of expected size (1,256 bp) was obtained. The nucleotide sequence of this was determined. It contained an open reading frame of 1,236 bp encoding a predicted protein of 412 residues, the molecular weight of which was calculated to be 44,156. The predicted amino acid sequence was similar to that of the OAH1 protein4) and OAH SHases of Thermus aquaticus,8) Aspergillus nidulans,10) Saccharomyces cerevisiae,11) and Candida albicans,12) with 43, 78, 38, 37, and 36% identity respectively, and to that of CTT γ-synthese of E. coli,13) with 32% identity. The identity of the four Thermus OAH SHases is summarized in Table 1. We have suggested that the oah1 gene is identical to the T. thermophilus HB27 MET17 gene, on the basis of an extremely high identity of deduced amino acid sequences of the two gene products.4) But the relation of the oah2 gene to the MET17 gene is unknown at present, because the identity between oah2 and MET17 was only 40%. In contrast, oah2 shows much higher identity to the T. aquaticus OAH SHase gene than to MET17, indicating that oah2 corresponds to the former gene. It is assumed that the Thermus species may have two kinds of OAH SHase genes intrinsically. Figure 1 shows alignments of the amino acid sequences of OAH1, OAH2, and T. aquaticus OAH SHase. The putative PLP-binding lysine residue was also estimated (Lys202).

The oah2 gene-expressing plasmid pEOAH2 was constructed, and was overexpressed in E. coli cells. An IPTG-induced band, observed at 44,000 Da in SDS-PAGE, was identical to the molecular mass calculated from the OAH2 sequence (data not shown). The results of purification are summarized in Table 2. SDS-PAGE showed that the preparation finally obtained had a purity of 95% or higher (data not shown). The subunit molecular weight of the gene product was estimated by SDS-PAGE to be approximately 44,000 (data not shown). This coincided well with the value calculated from the deduced amino acid sequence. The molecular weight of the purified protein in the native form was estimated to be about 190,000 by gel filtration (data not shown). Hence the native form of the enzyme is considered to be a tetramer of identical subunits.

The enzyme was shown to be active over a wide range of temperatures, and maximum activity was detected at about 70°C (data not shown). The effect of pH on activity was also investigated using a 100 mM K–P buffer (pH 6.0–8.0). The activity showed a bell-shaped curve against pH with a maximum at about pH 7.5, with 50% maximum activity at about pH 6.3 and pH 8.8 (data not shown). The effects of temperature and pH on the stability of the enzyme were also determined. After the purified preparation of the OAH2 protein was incubated at various temperatures for up to 60 min, activity was assayed using aliquots of the treated solutions. Stability of the enzyme at a high temperature was observed (80% activity remaining at 90°C for 60 min). The pH stability of the enzyme was investigated for a wide range of pH values (pH 3–13), at which it was incubated at 50°C for 30, 60, and 180 min after being diluted 10 times with a 50 mM GTA buffer. Enzyme activity was determined using 50 μl of the treated enzyme after it was diluted 5 times with a 50 mM K–P buffer (pH 7.8). It showed no loss of activity in the pH range of 7–12 (data not shown).

Among the carbonyl reagents tested, hydroxylamine inhibited the enzyme most sensitively (95% inhibition at 1 mM, 76% at 0.1 mM), phenylhydrazine moderately (95% at 10 mM, 12% at 1 mM), and semicarbazide was the most insensitive (76% at 10 mM, 12% at 1 mM). At a fixed sulfide concentration of 1 mM, a Km of 2.0 mM for OAH in the SHase reaction and a Vmax of 126 μmol/min/mg was obtained by Hanes–Woolf plotting (data not shown).

| The amino acid sequence identities of OAH1 (AB049221) and OAH2 (this study) of T. thermophilus HB8 (T.h HB8), MET1717 of T. thermophilus HB27 (T.h HB27), and OAH SHase (D87664) of T. aquaticus (T.aq) are represented as percentages. |
|---|---|---|---|---|
| T.th HB8 OAH2 | 40 | 40 | 78 |
| T.th HB8 OAH1 | 40 | 40 | 78 |
| T.th HB27 MET17 | 40 | 40 | 78 |
| T.aq OAH SHase | 40 | 40 | 78 |

Table 1. Comparison of Amino Acid Sequences of OAH SHases of Thermus Species
phoryl-L-serine) as substrates at a concentration of 5 mM. The enzyme also reacted with these amino acids, but to much lesser extents (data not shown). L-Methionine inhibited OAH SHase activity by 68% at a concentration of 1 mM. Propargylglycine, an active site-directed inhibitor of transsulfuration-catalyzing enzymes such as OAH SHase, inhibited OAH SHase activity by 72% at a concentration of 1 mM.

**Fig. 1.** Alignments of Amino Acid Sequences of *T. thermophilus* HB8 (T.th) OAH2 Protein, OAH1 (AB049221), and *T. aquaticus* (T.aq) OAH SHase (D87664).

Identical amino acid residues are indicated by shadows. The expected PLP binding lysine residues are indicated by a star.

**Table 2.** Summary of the Purification of the *oah2* Gene Product Overexpressed in *E. coli*

<table>
<thead>
<tr>
<th>Step</th>
<th>Volume (ml)</th>
<th>Total activity (units)</th>
<th>Total protein (g)</th>
<th>Specific activity (µmol/min/mg)</th>
<th>Yield (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude extract</td>
<td>500</td>
<td>25.2×10³</td>
<td>7.96</td>
<td>3.17</td>
<td>100</td>
</tr>
<tr>
<td>Heat treated (65°C)</td>
<td>430</td>
<td>19.6×10³</td>
<td>1.37</td>
<td>14.3</td>
<td>78</td>
</tr>
<tr>
<td>Q Sepharose pool</td>
<td>221</td>
<td>13.0×10³</td>
<td>0.361</td>
<td>36.0</td>
<td>52</td>
</tr>
<tr>
<td>Phenyl Sepharose pool</td>
<td>82</td>
<td>5.2×10³</td>
<td>0.077</td>
<td>67.7</td>
<td>21</td>
</tr>
</tbody>
</table>

Purification was performed with 50.4 g (wet weight) of cells as starting material. The DEAE-cellulose column previously employed in chromatography was replaced by a Q Sepharose Fast Flow (Pharmacia Biotech, Uppsala, Sweden) column (2.7 × 18 cm) for chromatography.
as CTT lyases and synthases, also inhibited activity by 89% at a concentration of 1 mM.

The biochemical and physico-chemical properties of the OAH2 protein so far investigated were very similar with those of the OAH1 protein, as summarized in Table 3. The former protein also showed a small amount of CTT\(^{-}/C13\)-synthase activity. Thus no clear difference was expected from the results obtained in this study between the roles of the two proteins. Why are two homologous genes, both encoding very similar OAH SHases, in the genome of this organism? What conditions under which the cell is placed cause expression of one of them or both to produce the protein(s)? In order to answer these questions, quantitative observation of expression of the two genes is needed. We are at present investigating to determine differences in the levels of expression of the two genes in cells fed with different sulfur sources, using the real-time PCR method.

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References


