Cloning of two Bombyx homologues of the Drosophila rosy gene and their relationship to larval translucent skin colour mutants

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Summary
To clone the Bombyx xanthine dehydrogenase (XDH) gene as a dominant marker for silkworm transgenesis, we performed nested reverse transcriptase–polymerase chain reaction (RT-PCR) using embryonic mRNA and primers designed from the conserved region of Drosophila and rat XDH genes. Sequencing of amplified 180 bp fragments showed that two different sequences were present in the fragments. Since both possessed striking similarity to XDH genes of other organisms, we considered these to be portions of silkworm XDH genes and designated them BmXDH1 and BmXDH2. Subsequently we cloned separately the entire region of the two cDNAs by PCR using phage DNA of an embryonic cDNA library and sequenced them. The two cDNAs were around 4 kb in size and possessed complete open reading frames. The deduced amino acid sequences of the two BmXDHs were very similar to each other and to those of other organisms. The expression pattern of wild-type larvae basically followed the tissue specificity of the enzyme and no significant difference was observed between the two XDH genes. The expression of both genes was detected in the XDH-deficient mutants, oq and og, but non-synonymous substitutions were specifically detected in the BmXDH1 of the oq mutant. In addition, a length polymorphism of the second intron of the BmXDH1 co-segregated with the oq translucent phenotype, suggesting that deficiency in BmXDH1 is the cause of the oq translucent phenotype.

1. Introduction
Construction of a system for transgenesis in the domesticated silkworm, Bombyx mori, is an important and urgent problem. This would be a powerful tool to study the mechanism of regulation of silkworm genes or to produce new races with specific characters, such as disease or high temperature resistance. To develop this system, cloning of a selectable marker gene to identify transgenic insects is crucially important and in this case visible marker genes would be highly preferable. For example, the rosy gene, which encodes the structural gene for xanthine dehydrogenase (XDH, EC 1.1.1.204) (Keith et al., 1987; Lee et al., 1987), is widely used as a selectable marker in Drosophila (Rubin & Spradling, 1983). However, no such marker gene has been cloned in the silkworm.

More than 320 mutant strains of silkworm are available, most of which carry morphologically visible mutations (Doira, 1978; Goldsmith, 1995). Among them three mutations were previously identified to be deficient in XDH activity, and this deficiency causes translucent skin in the mutant larvae (Tamura, 1977, 1983). Because uric acid is synthesized poorly or not at all, the larval skin, which normally accumulates uric acid granules and looks opaque and white, cannot accumulate the granules and becomes translucent (Tamura & Akai, 1990). Since the mutant larvae can easily be discriminated from wild-type larvae even at very early stages, we proposed that the combination of a normal XDH gene and such mutants would be useful to develop a marker-recipient system in the silkworm.

Thus, we decided to clone the XDH gene of B. mori. We report in this paper the isolation of two different XDH genes, designated BmXDH1 and BmXDH2. The sequences of the two full-length cDNAs indicate that they are similar but apparently different genes and similar to other known XDHs. Both partial sequencing of these genes of the XDH-deficient mutants and F2 linkage analysis strongly suggested
that mutations in BmXDH1 are the basis of the og mutant phenotype.

2. Materials and methods

(i) Silkworm stocks, mRNA purification and cDNA synthesis

The B. mori strain, C108, was used in these experiments unless otherwise stated. This strain and the og mutant were obtained from the Laboratory of Genetic Resources, National Institute of Sericultural and Entomological Science, Tsukuba. The mutants, og t and og, were generously provided by the Institute of Genetic Resources, Kyushu University. Purification of mRNA from embryos, fat bodies, midguts, malpighian tubes and silk glands was carried out with a QuickPrep micro mRNA purification kit (Pharmacia). The first-strand cDNA for polymerase chain reaction (PCR) was synthesized with a First-Strand cDNA Synthesis Kit (Pharmacia).

(ii) PCR amplification to obtain fragments of the XDH gene

The sequences of the oligonucleotides used as primers for PCR are shown in Table 1. First-strand cDNA synthesized from mRNA of 48 h embryos was used as a template for the first PCR reaction. The first PCR reaction was performed in a 25 µl reaction mixture containing 1 µl of synthesized embryonic cDNA, 1 µM primers, 1 U Taq polymerase (Promega), 0.2 mm of each of the dNTPs (Pharmacia), 50 mm KCl, 10 mm Tris-HCl (pH 9.0), 0.1% Triton X-100 and 2.5 mm MgCl2. The amplification was carried out with 10 min denaturation at 90 °C, followed by 10 cycles of denaturation (94 °C, 1 min), annealing (42 °C, 2 min) and extension (72 °C, 3 min), 10 cycles of [94 °C, 1 min; 52 °C, 2 min; and 72 °C, 3 min] and 10 cycles of [94 °C, 1 min; 55 °C, 2 min; and 72 °C, 3 min]. The second amplification was performed in the same reaction mixture as the first PCR except that 1 µl of the first PCR reaction was used instead of the first-strand cDNA reaction. This amplification was carried out with 2 min denaturation at 94 °C, followed by 30 cycles of [94 °C, 1 min; 42 °C, 2 min; and 72 °C, 3 min].

(iii) Two-step PCR amplification to clone the 5' and 3' unknown region

To clone the full-length XDH cDNA, an embryonic cDNA library was constructed from cDNA synthesized from mRNA of 48 h embryos using λgt11 (Stratagene). The library was divided into aliquots containing around 104 pfu, and transfected to E. coli, Y1088. After lysis, cell debris was removed by centrifugation. Then phage DNA was purified by phenol/chloroform extraction and ethanol precipitation and dissolved in 10-1 TE buffer. The PCR

Table 1. Oligonucleotide primers used in this experiment

| Name     | Sequence                  | Position of 5' end on BmXDH1 and BmXDH2 cDNA sequences |
|----------|---------------------------|-------------------------------------------------------|
| xdhFA    | TGYGGNACNAARYTNGGNTG      | 227(BmXDH1), 281(BmXDH2)                              |
| xdhFB    | GCNGTNACNACGNTNGARGG      | 271(BmXDH1), 325(BmXDH2)                              |
| xdhRA    | ATNGGNCKRTANCCNGTRCA      | 605(BmXDH1), 659(BmXDH2)                              |
| xdhRB    | CANARRTTNCYTGRAAANGC      | 581(BmXDH1), 635(BmXDH2)                              |
| lamF     | GGTGGCGACGACTCTGAGGACCCG  | lambda phage vector sequence                          |
| lamR     | TTGACACCAGACCAACTGTAATG   | lambda phage vector sequence                          |
| xdh1RA   | TACATGGACATTACGATTCC      | 512                                                   |
| xdh1RB   | GGGTACAGAAACCGCACTG      | 491                                                   |
| xdh2RA   | GCCACGCATTACCGCGATG      | 412                                                   |
| xdh2RB   | CGAAACCATTGACCGTTACAACG   | 360                                                   |
| xdh2RC   | GTTCCATTCCGGATCG         | 267                                                   |
| xdh1FA   | GAAAGTGGATGTTGTTCTTG     | 271                                                   |
| xdh1FB   | GGAATCTGAAATGTCAGTGA     | 492                                                   |
| xdh2FA   | GCACACCCAGGAATAGTCA      | 538                                                   |
| xdh2FB   | GAGGGTGGTTCCTAACAGGCA    | 609                                                   |
| xdh1QF   | CCAGGCAATCCTACAGAGA      | 2479                                                  |
| xdh1QR   | CCAGGTTCATCCTCATATG      | 3176                                                  |
| xdh2QF   | CTTGAGAGTCTGATTTTATTG    | 2157                                                  |
| xdh2QR   | CGGCAAAAGCCACACTCCA      | 2579                                                  |
| xdh1LF   | GTTGCCAGGAGTGGAGATGTTG   | 266                                                   |
| xdh1LR   | GTACGCTGAGAAGCGLGACT     | 783                                                   |

‘Y’ denotes T or C, ‘R’ denotes A or G, ‘K’ denotes G or T, and ‘N’ denotes A, C, G or T. All primers were synthesized with ABI Model 392 (Perkin-Elmer).
reaction was performed using this phage DNA solution as a template. The amplification of the 5' region was performed under the same conditions as the second PCR described above. A long PCR was carried out for the cloning of the 3' region. The first amplification was performed in a 25 µl reaction mixture consisting of 1 µl phage DNA, 1 µm primers, 1 U Takara Ex Taq polymerase (Takara shuzo, Japan) and 0-2 mm dNTPs in the accompanying buffer. The amplification was carried out with 2 min denaturation at 94 °C followed by 30 cycles of [94 °C, 30 s; 55 °C, 1 min; and 72 °C, 4 min]. The second PCR reaction was performed under the same conditions except for the substitution of 1 µl of the first amplified mixture for the phage DNA.

(iv) Determination of nucleotide sequence

Amplified PCR fragments were treated with T4 polynucleotide kinase (Nippon gene, Japan), blunted with Klenow fragment (Takara shuzo, Japan), and inserted into the Smal site of pUC19. When fragments were too large for insertion, endonuclease digestion was performed with Sau3AI or RsaI before ligation. DNA sequencing was carried out with these plasmid DNAs using an auto-sequencer (ABI 373A, Perkin-Elmer). The analysis of nucleotide sequence data, comparison of translated amino acid sequences and construction of phylogenetic tree were performed with GENETYX-MAC software (v. 8.5; Software Development, Japan).

(v) Detection of transcripts using RT-PCR

Reverse transcriptase–polymerase chain reaction (RT-PCR) was used to estimate the level of transcription. The first-strand cDNA synthesis was primed from random hexadeoxynucleotides and performed with a First-Strand cDNA Synthesis Kit (Pharmacia) in 10 µl reaction. Three hundred nanograms of poly-A RNA was used for each reaction and 0.33 µl of the completed reaction mixture was used for amplification. The amplification was carried out with 2 min denaturation at 94 °C, followed by 25, 27 or 29 cycles of [94 °C, 30 s; 55 °C, 1 min; and 72 °C, 2 min] using primers xdh1QF and xdh1QR for BmXDH1, and xdh2QF and xdh2QR for BmXDH2 (Table 1).

(vi) Linkage screening of the BmXDH1 gene

PCR primers, xdh1LF and xdh1LR, were designed to amplify the region containing the second intron of the BmXDH1 gene (Fig. 1, Table 1). The amplification was carried out with 2 min denaturation at 94 °C followed by 40 cycles of [94 °C, 1 min; 55 °C, 1 min; and 72 °C, 3 min]. Amplified products were isolated and checked by further PCR of an internal coding region.
Fig. 2. Comparison of XDH amino acid sequences. The amino acid sequences of BmXDH1, BmXDH2 and XDHs of *D. melanogaster* (Keith et al., 1987), *Calliphora vicina* (Houde et al., 1989), rat (Amaya et al., 1990) and chicken (Sato et al., 1995) were compared. The identical residues among them are shown with asterisks at the bottom. Conserved 2 Cys residues, putative 2Fe-2S-type redox centres, are underlined. Shaded regions indicate intermediate fragments of proteolytic digestion (Sato et al., 1995).
We estimated the probability that all individuals are homozygotic for particular locus by the formula \( p = (1-r)^n \), where \( p \) is probability, \( r \) is recombination frequency and \( n \) is number of individuals.

(vii) Nucleotide sequence accession numbers

The nucleotide sequence data reported in this paper will appear in the GSDB, DDBJ, EMBL and NCBI nucleotide sequence databases with the following accession numbers: D38159 for BmXDH1 and D43965 for BmXDH2.

3. Results

(i) Amplification of XDH homologous sequence

The complete cloning strategy for the silkworm XDH gene is shown in Fig. 1. When comparing the Drosophila and rat XDH genes, many regions of amino acid sequence are well conserved (Keith et al., 1987; Amaya et al., 1990). From these conserved regions, we designed four degenerated primers listed in Table 1. To obtain the first short fragment of the Bombyx XDH gene, we performed nested PCR using the primers xdhFA and xdhRA for the first amplification, and xdhFB and xdhRB for the second PCR. As a result, a fragment of about 180 bp consistent with our expectations was amplified from the second PCR and inserted into the plasmid pUC19. Sequencing of several of these pUC19 clones showed that two apparently different types of insertions were present. Since these two types of sequences possessed striking similarity to the sequences near the 5' end of the XDH genes of other organisms (data not shown), the cloned fragments were regarded to be the corresponding sequences of independent XDH genes of B. mori. Therefore, we named these two genes BmXDH1 and BmXDH2.

(ii) Determination of 5' and 3' unknown sequences

We failed to obtain XDH clones from the embryonic cDNA library using the amplified fragments as probes. This is probably because the size of the fragments was too small. To obtain the full-length cDNA sequences, we performed direct PCR of phage DNA extracted from the cDNA library as shown in Fig. 1, and primers were designed from the sequenced region and both arms of \( \lambda gt1 \).

With regard to the cloning of the 5' unknown region of BmXDH1, we used the primers lamF and xdh1RA for the first amplification and then lamF and xdh1RB for the second PCR. In this PCR, we were able to clone the region containing the putative initiation codon. Except for BmXDH2, the amplified products were too short. Therefore, upstream primers (xdh2RC) were newly synthesized and similar PCR was performed. Finally, the fragment including the putative initiation codon was obtained and sequenced.

The 3' unknown regions were too large for ordinary PCR. However, long PCR for amplifying more than 10 kb was recently reported (Barnes, 1994; Chen et al., 1994). We used this method to obtain the 3' regions using the same strategy. When using ordinary \( Taq \) polymerase in the protocols for the amplification of short fragments, we could find no specific products after performing nested PCR of xdh1FA and lamR, followed by xdh1F2 and lamR or xdh2FA and lamR, and then xdh2FB and lamR. In contrast, 3' regions of BmXDH1 and BmXDH2 were successfully amplified with a specially prepared \( Taq \) polymerase for long PCR in its accompanying buffer by using the same nested PCR and primers.

Both amplified products of BmXDH1 and BmXDH2 were around 3-5 kb in size. This is too large to sequence directly; therefore, we digested the fragments with Sau3A1 or RsaI and inserted the digested fragments into plasmids. These clones were sequenced, and the gaps between clones were filled with the sequences obtained from the fragments amplified directly from cDNA or genomic DNA.

Partial genomic sequencing of this 5' portion revealed the positions of two introns in BmXDH1 (nucleotide positions between 182 and 183, 336 and 337), and five introns in BmXDH2 (nucleotide positions between 234 and 235, 387 and 388, 2499 and 2500, 2808 and 2809, 3142 and 3143). The position of the first intron was conserved among BmXDH1, BmXDH2 and known Diptera XDH genes, but the second intron of the Bombyx genes was located just downstream from the first intron, unlike in Diptera.

Beginning with identical putative initiation codons, we confirmed the largest open reading frames of 4068 bp in BmXDH1 and 4005 bp in BmXDH2 (data not shown), respectively. We found that these two open reading frames possessed significant similarity throughout the region with other organisms, but similarity between BmXDH1 and BmXDH2 was not so remarkable (57.3 % nucleotide identity and 55.8 % amino acid identity) as compared with other organisms (49.9–58.8 % amino acid identity). The phylogenetic tree showed that the divergences of XDH genes are generally consistent with the known phylogeny of mammals, birds, Diptera and Lepidoptera (Fig. 3).

(iii) Tissue specificity of BmXDH1 and BmXDH2 gene expression

Since BmXDH1 and BmXDH2 were cloned from mRNA of embryos 48 h after egg laying, both were transcribed in embryos at this stage. To examine whether there is tissue specificity for these two genes,
RT-PCR was carried out on poly-A RNA extracted from fat body, midgut, malpighian tubes and silk glands (Fig. 4). As expected, expression of both XDH genes was observed in fat body, midgut and malpighian tubes, but was quite low in silk glands. It seemed that the level of transcription was higher in malpighian tubes and lower in the midgut (lanes 1–9). This expression pattern was in basic agreement with the observation of the distribution of enzyme activity in larval tissues (Hayashi 1961a, b; Tamura, 1983). We found no significant difference in tissue specificity of BmXDH1 and BmXDH2 at the resolution of our assay.

(iv) Non-synonymous substitutions detected in BmXDH1 of oq

We also analysed XDH gene expression in og and oq mutants. The level of the two BmXDH gene transcripts was assayed by RT-PCR of poly-A RNA prepared from fat body of homozygotes and heterozygotes of og, og and oq, an allele of og. The transcriptional level of the XDH genes in these mutants was not so different from that of wild type at the resolution of our assay (data not shown). However, we often found aberrant products when BmXDH1 cDNA was amplified from mRNA of the og mutants (data not shown), suggesting the possibility that some mutations might have occurred in BmXDH1 of the og mutants. Consequently, we sequenced the 5’ portion of the cDNA (nucleotide positions between 1 and 1679) from og homozygotes and heterozygotes. Sequences of heterozygotes showed a clear dimorphism and one sequence was coincident with that of the homozygotes. Sequencing of an extended region confirmed the dimorphism of heterozygotes. We found three non-synonymous and 21 synonymous substitutions in the homozygous type sequence and one non-synonymous and nine synonymous substitutions in the other type. We also performed partial sequencing of the BmXDH1 and BmXDH2 genes from og and og mutants, but there were no non-synonymous substitutions (data not shown). These results suggested that the BmXDH1 gene is concerned with the og mutation.

(v) Polymorphism in length of the BmXDH1 second intron co-segregated with the oq phenotype

To determine the relationship between the BmXDH1 gene and the og mutation, we performed an F2 linkage

Fig. 4. Expression of BmXDH1 (A) and BmXDH2 (B) in various tissues. RT-PCR amplification was stopped at the 25th, 27th or 29th cycle. Lanes 1, 4, 7, 10 and 13, PCR products of 25 cycles; lanes 2, 5, 8, 11 and 14, 27 cycles; lanes 3, 6, 9, 12 and 15, 29 cycles.
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Fig. 5. Co-segregation of a BmXDH1 polymorphism and oq phenotype. Primers xdh1LF and xdh1LR were designed to amplify the second intron inserted at the nucleotide position between 336 and 337 of BmXDH1 cDNA. (A) Polymorphism in the length of the second intron in the BmXDH1. Template DNAs were prepared from 50 larvae per strains. (B), (C) Amplification of the polymorphic fragment from genomic DNAs of 15 translucent (B) and normal skin colour (C) F₂ individuals. Bulked DNA indicates a product generated from genomic DNA of 50 normal skin colour F₂ insects.

analysis based on PCR. We used bulked genomic DNAs prepared from 50 larvae per strain for screening of polymorphisms. Primers xdh1LF and xdh1LR, encompassing the second intron of the BmXDH1 gene, showed a clear length polymorphism between the oq mutants and two wild-type strains, C108 and Daizo (Fig. 5A). Compared with wild-type strains, which had a single 920 bp fragment, the oq homozygotes had a longer single 1200 bp fragment. Both fragments were observed in the F₂ progenies showing normal skin colour, a mixture of oq/+oq and +oq/+oq.

Similar heterozygosity was also detected in the oq mutants, but there was no difference between mutant and normal skin colour populations (Fig. 5A).

Genomic DNAs were isolated from 30 larvae of an intercross of oq/+oq. Fifteen of them were translucent and the rest were of normal skin colour. Following identical PCR amplification, all the translucent individuals had single longer fragments without exception (Fig. 5B). The probability of this event was 18.4% when BmXDH1 was 5 cM from the oq locus, and 54.5% when BmXDH1 was 2 cM from it. If bulked
DNA from 50 larvae were taken into consideration, these values were 0.0652 and 7.23%, respectively. In contrast, five of 15 normal skin colour insects had a single shorter fragment, and the rest had both fragments. The ratio was identical to 1:2, the expected ratio of + "oq"/+ "oq" to og/+ "oq" (Fig. 5 C). These results strongly suggested that BmXDH1 and the og locus are closely linked.

4. Discussion

We have determined the full-length cDNA sequence of two *Bombyx* xanthine dehydrogenase genes (XDH) from embryonic mRNA. This is the first report of cloning and sequencing of XDH in lepidopterous insects. XDH plays very important roles in nitrogen metabolism. The final nitrogenous excretory product in most Lepidoptera is uric acid, and the enzyme catalyses the conversion of hypoxanthine to xanthine and xanthine to uric acid (Bursell, 1967; Cochran, 1975). The enzyme activity and amount of uric acid are influenced by nutritional conditions in the silkworm (Ito & Murakami, 1964; Horie & Watanabe, 1983). Purification of this enzyme from *Manduca sexta* reveals that there are specific cellular localizations and levels of enzyme during development (Buckner et al., 1993). Cloning of the XDH gene would facilitate studies on these physiological roles and regulation mechanisms in Lepidoptera.

In our study we found two *Bombyx* XDH genes. Both genes possessed significant similarity with those of other organisms, including flies (Keith et al., 1987; Lee et al., 1987; Houde et al., 1989; Riley, 1989), chicken (Sato et al., 1995) and mammals (Amaya et al., 1990; Terao et al., 1992). Sato and colleagues proposed that XDH were composed of three domains: the N-terminal iron–sulphur centres, the intermediate FAD associating domain, and the C-terminal molybdenum centres (Sato et al., 1995). Both *Bombyx* genes have putative 2Fe/2S-type redox centres (Fig. 2, underlined 2 Cys residues). They also reported that the hinge region of the N-terminal and the intermediate domains were highly sensitive to subtilisin digestion (Sato et al., 1995). It coincides with very low similarity in the region (Fig. 2).

No organisms have been reported to date to have two or more functional XDH genes. A phylogenetic tree of XDH genes suggested that the divergence of BmXDH1 and BmXDH2 was later than that of vertebrates and invertebrates but earlier than that of Lepidoptera and Diptera (Fig. 3). BmXDH1 seems to be a member of the previously known Diptera *rosy* gene family and BmXDH2 is fairly remote from them (Fig. 3). However, this cannot explain the fact that no other insects have been reported to have multiple XDH genes or pseudogenes. One speculation is that they exist but have just not been discovered yet. Alternatively, BmXDH1 and BmXDH2 diverged after Lepidoptera and Diptera were differentiated but functional differences between them make genetic similarity lower than expected. Further studies on other insects will be required to solve this problem.

We could not find a functional difference between BmXDH1 and BmXDH2 so long as we only examined tissue specificity in wild-type larvae. Both BmXDHs were highly expressed in midgut, but not in the silk glands (Fig. 4); similarly, transcription of BmXDH1 and BmXDH2 were observed in fat bodies from og and og homozygotes (data not shown). However, expression levels of the BmXDH1 mRNA in og homozygotes are reported to be lower than in normal skin colour insects (N. Komoto et al., unpublished data).

Our primary interest is to develop a marker-recipient system for transgenesis in the silkworm. For this purpose, identifying a visible mutation and defining its underlying basis is the most important objective. Upon examining RT-PCR products in more detail, we found that none of the sequences was inserted or deleted. Instead, we detected an evident dimorphism in sequences of clones from og heterozygotes, one of which was identical to that found in og homozygotes. Moreover, polymorphism in the length of the second intron of BmXDH1 was co-segregated with the og phenotype (Fig. 5). These results strongly suggest that some deficiency in the region around the BmXDH1 gene is the cause of the og translucent phenotype.

However, it remains unclear whether the base substitutions and the intron length polymorphism reported in this experiment are the result or the cause of the mutation. Most base polymorphisms were located on the hinge and intermediate domain where the functional requirements seemed to be relatively low (Sato et al., 1995). We did not succeed in detecting any alternative splicing related to the second intron. Additional studies are needed, including sequencing extended regions of mutants and site-directed mutagenesis.

Further analyses are required to characterize these mutations; however, our results suggest that a combination of functional BmXDH1 together with the og mutant is a promising candidate for a marker-recipient transgenic system for the silkworm. We are now trying to isolate intact clones of BmXDH1 and BmXDH2 and to construct a transfer vector to introduce into mutant silkworms by a microinjection or particle delivery system.

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