Original Article

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Silkworm arylsulfatase in the midgut content is expressed in the silk gland and fed *via* smearing on the food from the spinneret

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SUMMARY We found the activity of arylsulfatase in the midgut contents of the silkworm, *Bombyx mori*. We identified a 60-kDa protein that comigrates with the activity on a column chromatography following ammonium sulfate precipitation. Based on its partial amino acid sequence, we searched for its coding gene using Basic Local Alignment Search Tool (BLAST) and identified *KWMTBOMO05106*. Transcriptional data suggest a specific expression of the gene in middle silk glands. The majority (80%) of arylsulfatase activity was found in the silk glands, concurring the specific transcription in the silk gland. Observing the feeding behaviour of the silkworm, we found that silkworms smear a mucus secretes from the spinneret on the food pellet as they feed on. Arylsulfatase activity was also detected in the food pellet bitten by the silkworm as well as in the gut content. Furthermore, arylsulfatase activity was not detected either in the food pellet and in the gut content when silkworms had obstructed the spinneret. These results suggest that arylsulfatase is secreted from the silk glands and may contribute to digestive function.

Keywords silkworm, enzyme, secretion, arylsulfatase

1. Introduction

In previous work, we have proposed the utility of the silkworm as an experimental animal for evaluating the therapeutic potential of pharmaceuticals (*1*). We have reported that the therapeutic potential of existing therapeutic agents can be quantitatively assessed in a silkworm bacterial infection model, obtaining 50% effective dose (ED_{50}) values comparable to those in mammalian models (*2,3*). In addition, exploration using soil bacterial culture supernatants (*4*) has led to the discovery of novel antibiotics, such as lysocin E (*5,6*) and ASP2397 (*7*), which have been shown to have a therapeutic effect in mice models. This is partially because the pharmacokinetic parameters of antimicrobials are well matched between the silkworm and mammalian models (*8,9*). With regard to metabolism, which has a major impact on the pharmacokinetics of compounds, it has been shown that in the silkworm, as in humans, hydroxylation by cytochrome P450s occurs, followed by conjugation reactions and excretion (*8,10*). With regard to the conjugation reactions in the silkworm, glycosyltransferases(*11,12*), glutathione transferases (*13*), and sulphotransferases (*14*) have been identified. Although these conjugation enzymes have been studied in the silkworm, little work has been performed on the metabolism of the conjugated compounds, although a group of conjugation enzymes involved in human pharmacokinetics has been identified in the silkworm.

A typical example of the metabolism of conjugated compounds is the reaction by arylsulfatases. Arylsulfatases are a group of enzymes that catalyze the desulfation of aromatic sulfates (*i.e.*, aryl sulfates) (*15*). In the human gut, arylsulfatases of enterobacterial origin are known to affect the metabolism and kinetics of steroid hormones that are sulfate-conjugated and excreted with the bile (*16,17*). A deficiency of arylsulfatase in humans causes metachromatic leukodystrophy due to the accumulation of sulfatides, resulting in central and peripheral nerve damage (*18*). Arylsulfatases are conserved across animal phyla (*15*), and the presence of arylsulfatase invertebrates has been reported in the literature (*19*). In *Caenorhabditis elegans*, a deficiency of arylsulfatase, which is involved in the sulfation of steroid hormones, has been shown to prolong lifespan (*20*), but little is known about its physiological function. Also in the silkworm,

arylsulfatase expression in the brain and other organs has been reported, yet its physiological function is unknown (*21*). We performed an assay using the model compound 4-methyl umbelliferyl sulfate and found arylsulfatase activity in the silkworm gut contents.

In the present study, we purified the arylsulfatase in the intestinal tract and identified the primary structure of the protein. We further showed that this enzyme is secreted from the silk gland onto the food pellet during feeding, which may indicate that the enzyme contributes to metabolizing ingested materials. These results shed light on the hitherto unknown physiological function of arylsulfatase in the silkworm

2. Materials and Methods

2.1. Raring of silkworm

Silkworm eggs (Hu•Yo × Tsukuba•Ne) were purchased from Ehime Sanshu (Ehime, Japan) and hatched larvae were fed artificial food, Silkmate 2S (Katakura Industries, Co., Ltd. Tokyo, Japan) at 27˚C until the 5th instar stage.

2.2. Arylsulfatase assay

4-methyl umbelliferyl sulfate (1.3 mM) was mixed with a sample in a total of 150 µL of 50 mM Tris buffer pH 7.9 and incubated at 30˚C for 30 min. After adding saline 350 µL and methanol 500 µL, the samples were centrifuged at 4˚C, 14,000 rpm for 5min. The 200 µL of supernatant was mixed with 3 mL of 1.6 M glycine buffer (pH 11) and the fluorescence of umbelliferon, desulfated from 4-methyl umbelliferyl sulfate, was measured with the fluorometer (Hitachi F4500, Tokyo, Japan).

2.3. Purification of arylsulfatase from silkworm digestive content

Silkworm intestine content was collected from the 5th instar larva (Day 3 to 6) and homogenized (Fr. I). The samples were centrifuged at 4˚C, 16,000× *g* for 20 min, and precipitants were collected (Fr. II). The Fr. II was homogenized with 50 mM ammonium sulfate and supernatants were collected by centrifugation at 4˚C, 16,000× *g* for 20 min (Fr. III). Next, ammonium sulfate was added to the supernatant (final 3.5 M) and incubated at 4˚C for overnight. And then, centrifuged at 4˚C, 16,000× *g* for 20 min and dissolved in 50 mM Tris-HCl (pH 7.9) (Fr. V). To prepare Fr. VI, the samples were mixed with an equal volume of acetone and froze at – 20˚C for 1 h and centrifuged at 125,000 × *g*, 4˚C for 30 min. The precipitants were dissolved in 50 mM Tris-HCl (pH 7.9) with 20% glycerol by sonication (Sonifire OPC × 15 sec) and centrifuged again at 125,000× *g*, 4˚C for 30 min. The supernatants (Fr. VI) were applied onto diethylaminoethyl (DEAE)-cellulose column chromatography and collected flow through fr. (Fr. VII). Further, MonoS column chromatography was performed by a linear gradient of 0 to 0.5 M ammonium sulfate.

2.4. Gene expression analysis by quantitative RT-PCR

Total RNA was prepared from each tissue using the RNeasy mini kit (Qiagen, Venlo, Netherlands) and after DNase treatment, cDNA was prepared by reverse transcription reaction (High-Capacity RNA-to-cDNA Kit, Applied Biosystems) using 50 ng of total RNA. The prepared cDNA was used as a template for quantification of the arylsulfatase gene by real-time PCR (Thunderbird SYBR qPCR Mix from Toyobo (Japan)) by using 7500 Fast Real-Time PCR System (Applied Biosystems). The primer sequences used for quantification were 5'-CCACTTAGCAACAGCGAGGA-3' and 5'-TCAGTTCGTGATTCGCCGAT -3'.

2.5. Bioinformatics analysis

Sequence reads data of RNA-Seq analysis in silkworm organs (DRR142721, DRR142719, DRR077426, DRR077427, and DRR12883) were downloaded from Sequence Read Archive (SRA). RNA-Seq analyses were performed using CLC Genomics Workbench ver. 21 (Qiagen, Venlo, Netherlands). Reads were aligned to the p50T strain genome (Accession No. GCF_014905235.1) allowing a minimum length fraction of 0.8 and a minimum similarity fraction of 0.8. Transcripts per million (TPM) values were normalized by trimmed mean of M values (TMM) method.(*22*) Calculation of phylogenic tree was performed at the site *http://www.phylogeny.fr/simple_phylogeny.cgi* by one-click mode (*23*). Perdition of signal peptide of protein was performed by SignalP ver. 6.0 (*24*).

3. Results

3.1. Purification and identification of arylsulfatase from the silkworm intestine

We measured the activity of arylsulfatase by the fluorescence intensity of umbelliferon produced by the enzymatic activity from the non-fluorescent substance 4-methylumbelliferone sulfate (*25*). As a result, arylsulfatase activity was identified in the silkworm gut contents. As this activity was recovered in centrifuged pellets, the responsible enzyme appears insoluble. We therefore searched for conditions where the enzyme could be solubilized and found that 0.5 M ammonium sulfate could solubilize the enzyme (Table 1). When the concentration of ammonium sulfate was increased to 3.5 M, activity was detected in the precipitate. Partial purification of the enzyme by acetone precipitation and DEAE cellulose chromatography was then performed,

Table 1. Purification table of arylsulfatase from silkworm digestive content

Fraction		Sulfatase activity (U × 10^3)	Protein (mg)	Specific activity (U × 10^3/mg)	Yield (%)
I	Gut content	910	8200	0.11	100
II	Gut content ppt	860	4200	0.20	95
III	Gut content ppt wash	670	4000	0.17	74
IV	Soluble sulfatase	680	650	1.0	75
V	3.5M $(NH_4)_2SO_4$ ppt	600	600	1.0	66
VI	Acetone treatment ppt	400	360	1.1	44
VII	DEAE flow through	150	140	1.1	16

and activity was recovered in the flow-through fraction of DEAE cellulose column chromatography. The yield from the intestinal contents was 16%, with a 10-fold increase in specific activity (Table 1). Furthermore, the DEAE flow-through fraction was separated by Mono-S column chromatography, yielding a 60-kDa protein whose activity pattern matched the band intensity in SDS-PAGE.

Further analyzing the DEAE cellulose fraction, we performed Mono-S column chromatography using the DEAE cellulose fraction, and a major activity peak appeared (Figure 1A). This active fraction was analyzed by SDS polyacrylamide gel electrophoresis (SDS-PAGE) and several proteins were detected. Among them, a 60-kDa protein matched the pattern activity of arylsulfatase (Figure 1B). We then analyzed the partial amino acid sequence of the 60-kDa protein. The protein was extracted from the gel slice containing this band and the N-terminal amino acid sequence was analyzed by Erdmann degradation. As a result, TKRKSSNIVLIVADDL was identified. A BLAST search using this sequence in the Silkbase site (*https://silkbase.ab.a.u-tokyo.ac.jp/*), a database constructed by high-precision whole-genome sequencing, revealed a 100% match with the amino acid sequence encoded by the KWMTBOMO05106 gene (Figure 2A). Currently, five genes encoding arylsulfatases have been identified in the silkworm genome database (Figure 2A, Supplementary Table S1, *http://www.ddtjournal.com/action/getSupplementalData.php?ID=125*). This protein showed the highest homology to human arylsulfatase B and will henceforth be denoted as silkworm arylsulfatase B (Figure 2C). The amino acid sequence predicted from genome database sequencing (*26*) indicates that a signal sequence (predicted by SignalP 6.0) comes N-terminus region by following the amino acid residues that match the identified partial sequence. The amino acid sequence of the active domain, which is commonly conserved in arylsulfatases, was also conserved in this protein (*i.e.*, silkworm arylsulfatase B). Analysis of organ-wise expressions of this gene using silkworm RNA-Seq data registered in the SRA database showed that expression levels were high in the silk gland but low in the midgut (Figure 2D). This expression pattern was also confirmed by qRT-PCR (Figure 2E). Furthermore, when arylsulfatase activity was measured

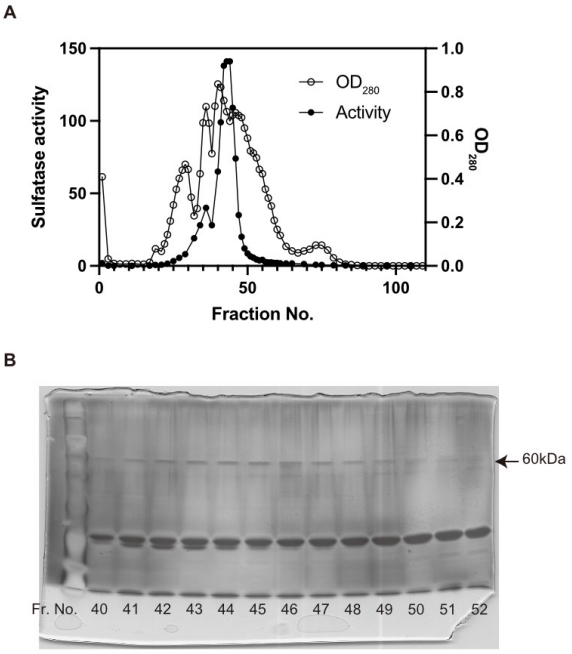

Figure 1. Elution profile of arylsulfatase activity in MonoS column chromatography. A. Protein and sulfatase activity elusion pattern from MonoS column chromatography. Protein concentrations were measured optical density of 280 nm of eluted fractions. Sulfatase activity was defined as the amount of umbelliferon production (nmol) in 20 μL aliquot of the assay sample. **B**. Silver staining image of SDS-PAGE for fractions 40 to 52 of MonoS column chromatography.

for each organ, the major activity was found in silk glands (Table 2).

3.2. Dynamics of silkworm arylsulfatase B

During feeding, silkworms secrete materials from the spinnerets to the feeding area of mulberry leaves (*27*). Our observation of silkworm feeding behavior confirmed that silkworms secrete mucus from the spinnerets during feeding (Figure 3A and Supplementary Movie, *http://www.ddtjournal.com/action/getSupplementalData.php?ID=126*). We also found that arylsulfatase activity appeared on the mulberry leaves, when bitten by the silkworm (Figure 3B). Furthermore, we found that the spinnerets obstructed with a sticky bond resulted in the loss of arylsulfatase activity in the gut (Figure 3C).

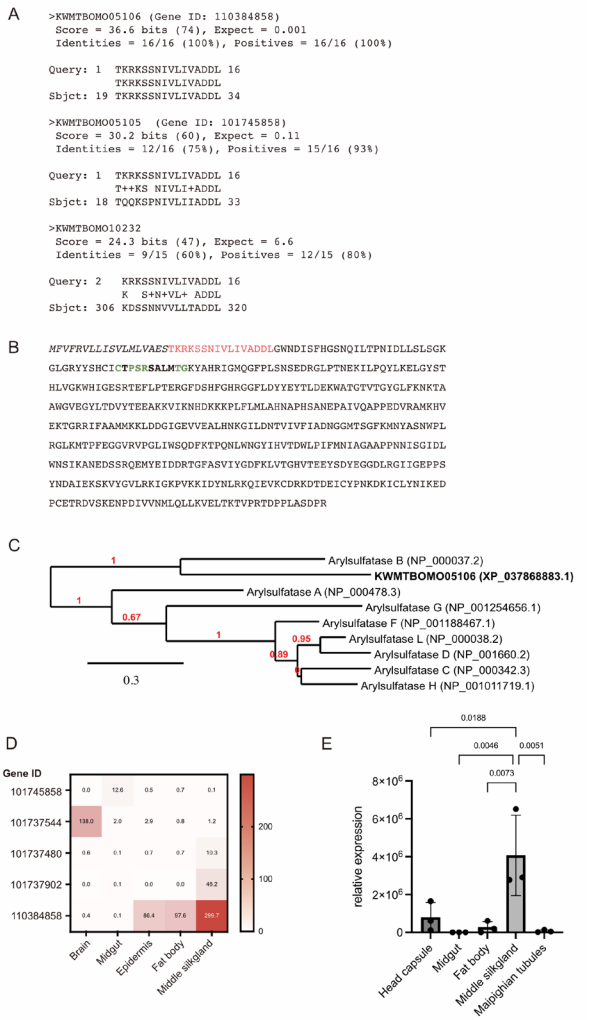

Figure 2. Identification and expression analysis of arylsulfatase in silkworm gut content. A. The result of blastP search using obtained amino acid sequence. **B**. Amino acid sequence of the product of the KWMTBOMO05106 gene. Red letters of amino acid sequence indicated identified by partial amino acid determined by Edman sequence. Green letters stand for the conserved motif of sulfatase activity. Italic letters indicated signal peptide estimated by SignalP 6.0. **C**. Phylogenic tree of KWMTBOMO05106 gene product compared with human arylsulfatases. **D**. RNA-Seq result of the genes annotated as arylsulfatase B in silkworm genome. **E**. The mRNA expression of the KWMTBOMO05106 gene (Gene ID: 110384858) in various organs was analyzed by RT-PCR.

4. Discussion

We have shown that arylsulfatase activity is present in the silkworm intestinal tract, and it seems most likely that the arylsulfatase is produced in the silk glands, secreted from the spinneret during feeding, and then ingested into the gut. In this study, it was shown for the first time that a metabolic enzyme (*i.e.*, silkworm arylsulfatase B) for xenobiotics is expressed in the silk gland and secreted from the spinnerets and transported to the intestinal tract. As this enzyme is expressed in the silk gland, it may be possible that the presence of the enzyme may influence the composition of the silk glands, but so far there are no reports of sulfated

Table 2. Majority of arylsulfatase activity existed in the silk gland

Tissue	Sulfatase activity (U/tissue)
Silk gland	8,000
Muscle	2,600
Intestine content	2,000
Head	490
Hemolymph	250
Midgut	190
Fat body	120

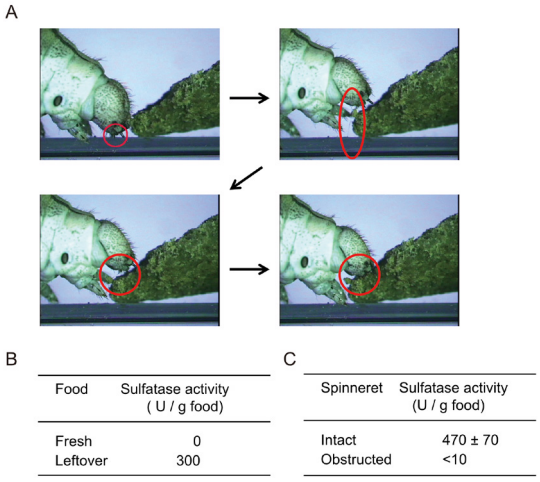

Figure 3. Silkworm Arylsulfatase B was secreted from the spinneret and transferred into the gut *via* feeding. A. Silkworm secret mucus from the spinneret and feeds on mucus-coated food. **B**. Arylsulfatase activity in food left uneaten by silkworms. **C**. Arylsulfatase activity in the intestine is reduced by obstruction of the spinneret (n = 3, data are shown as mean ± S.D.).

compounds in the silk glands that can be degraded by arylsulfatase. It is therefore likely that the physiological function of arylsulfatase B may be a digestive enzyme, and it works after being secreted from the spinneret, but not in the silk gland. In the literature, enzymes in mucus secreted from the spinneret are known to inhibit the biosynthesis of green lead volatiles (an attractant for other insects) (*27*). In plants, sulfate conjugates are produced in flavonoid metabolism by sulfotransferases. Thus, it may be interesting to focus on the interaction between the secreted arylsulfatase and plant-derived substances. In addition to arylsulfatase, other enzymes such as α-glycosidase, lipase, and lysozyme were highly expressed in the silk gland (Supplementary Table S2, *http://www.ddtjournal.com/action/getSupplementalData.php?ID=125*). This suggests the presence of a group of enzymes with functions similar to arylsulfatase B. Nevertheless, silkworms covered the spinneret with sticky bonds showed no change in weight gain when fed artificial feed or mulberry leaves. Therefore, the significance of this enzyme for short-term growth may be limited, and the nutritional impact of the enzyme in the silkworm needs further

investigation.

In humans, arylsulfatase is involved in the metabolism of steroid hormones. Similarly, sulfotransferases catalyze sulfate conjugation reactions of juvenile hormones, ecdysone, and 20-hydroxysteroids in several insect species (*28*), but sulfate conjugates are rarely detectable in the body of insects (*29*). Based on the present finding, it may be because the sulfated steroid hormones in the intestine are rapidly metabolized to non-sulfated form by the arylsulfatase in the intestinal tract.

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