



Accumulation of 1-deoxynojirimycin in silkworm, *Bombyx mori* L.

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Abstract: 1-deoxynojirimycin (1-DNJ) contents in the silkworm, *Bombyx mori*, at different developmental stages and tissues were investigated by using reverse-phase high-performance liquid chromatography. The 1-DNJ contents of silkworm larvae change significantly with their developmental stages. The male larvae showed higher accumulation efficiency of 1-DNJ than the females and also a significant variation was observed among the silkworm strains. The present results show that tissue distribution of 1-DNJ was significantly higher in blood, digestive juice, and alimentary canal, but no 1-DNJ was observed in the silk gland. Moreover, 1-DNJ was not found in silkworms fed with artificial diet that does not contain mulberry leaf powder. This proves that silkworms obtain 1-DNJ from mulberry leaves; they could not synthesize 1-DNJ by themselves. The accumulation and excretion of 1-DNJ change periodically during the larval stage. There was no 1-DNJ in the newly-hatched larvae and 1-DNJ was mainly accumulated during the early and middle stages of every instar, while excreted at later stages of larval development. Further, it is possible to extract 1-DNJ from the larval feces and it is optimal to develop the 1-DNJ related products for diabetic auxiliary therapy.

Key words: Silkworm, 1-deoxynojirimycin (1-DNJ), Accumulation, *Bombyx mori*

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1 Introduction

Nojirimycin was firstly discovered from streptomycete. 1-deoxynojirimycin (1-DNJ) is the product of nojirimycin through hydrogenation. The natural DNJ was firstly isolated from mulberry tree (Yoshikaki and Hivonu, 1976), and up to now more than 20 of polyhydroxy alkaloids were identified from mulberry and silkworm (Asano *et al.*, 1994a; 1994b; 2001). As a piperidine alkaloid, DNJ is known as a highly effective α -glycosidase inhibitor (Yoshikuni, 1988; Yoshikuni *et al.*, 1988; Hughes and Rudge, 1994), and is one of the effective chemicals for anti-hyperglycemia. Presently DNJ and its analogs have been abstracted from a wide range of plants and

microbes (Asano *et al.*, 1998; 2000; Kim *et al.*, 1999), but the DNJ contents in mulberry tree are the highest compared with other plants (Kimura *et al.*, 2007; Yatsunami *et al.*, 2008).

Mulberry has been utilized as Chinese medicine against diabetes mellitus for long time. Ryu *et al.* (1997) firstly reported that the silkworm larval powder of the 5th instar (prepared by lyophilization) had a good effect on diabetic patients (Ryu *et al.*, 1997; 1999), and the effect for lowering the blood sugar level was further proved by subsequent research (Han *et al.*, 2007). Recently in Korea, Japan, and China these related products made of mulberry and silkworm larvae are popular for auxiliary therapy on diabetes mellitus.

The silkworm powder has higher DNJ content than mulberry, indicating that the larvae can accumulate and enrich DNJ from mulberry leaves (Kim *et*

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al., 2003), and thereby the larval powder displays more effective results on diabetic. However, it is unknown whether the silkworm itself synthesizes DNJ, and little is known about assimilation, enrichment, and excretion of DNJ in silkworm, as well as the factors affecting the DNJ accumulation. Our study provides a basis for the DNJ enrichment and excretion in the silkworm, which may play an active role for preparing DNJ-related products.

2 Materials and methods

2.1 Materials

Silkworm larvae were reared by feeding fresh mulberry leaves or artificial diet at 25 °C. Artificial diets were prepared (Table 1), and six types of diets containing various percentages of mulberry leaf powder were used. The dry diets were mixed with two-fold water and autoclaved.

2.2 DNJ measurement

For DNJ analysis, the silkworm larvae were collected after starvation for 24 h, and the samples were lyophilized, smashed, and passed through a 100-mesh screen. The artificial diets and mulberry leaves were dried under 60 °C, grounded, and passed through a 100-mesh screen.

DNJ content was measured according to the method reported by Kim *et al.* (2003). DNJ in the silkworm larva or mulberry leaf powder was extracted with 0.05 mol/L HCl, treated with 9-fluorenylmethyl (FMOC) in pH 8.5 borate buffer solution to produce DNJ-FMOC complex compound, and finally detected by ultraviolet-reverse-phase high-performance liquid chromatography (UV-RP-HPLC).

1. Preparation of DNJ crude extract: About

30 mg of larva or mulberry leaf powder was mixed with 1 ml of 0.05 mol/L HCl, followed by vortexing for 1 min, and then treated with ultrasonic for 40 min. After centrifugation at 12000 r/min for 30 min, the supernatant was collected. The pellet was treated again by repeating the above steps, and then supernatants were combined and diluted to 2 ml by adding distilled water. Thus obtained samples were used as crude extract.

2. DNJ derivation: The crude extract (35 µl of larval powder crude extract and 140 µl of mulberry leaf powder crude extract) was mixed with 169 µl of 0.4 mol/L potassium borate buffer (pH 8.5) in a 1.5-ml microtube. 20 µl of 5 mmol/L 9-fluorenylmethyl chloroformate (FMOC-Cl) dissolved in 50% (v/v) CH₃CN was added, and mixed thoroughly. The mixture was placed in 20 °C water bath for 20 min, and subsequently 25 µl of 1 mol/L glycine was added to quench the remaining FMOC-Cl. Finally, 66 µl of 0.1% (v/v) acetic acid was added to stabilize the DNJ-FMOC formed in the reaction, and distilled water was added to final volume 707 µl. The sample was ready for DNJ measurement by filtering through a 0.2-µm nylon syringe filter.

3. DNJ determination: RT-HPLC was applied for DNJ content determination. Dionex 500E HPLC was procured from Dionex Co. (USA). DNJ-HCl was obtained from Sigma Chemicals (USA), and FMOC-Cl was from Fluca. HPLC system consisted of a VENUS-C18-5u column (250 mm×4.60 mm i.d.), a UVD170U detector (excitation 254 nm), and a Dionex Chromeleon working station as data processing software. The column was eluted with a mobile phase of acetonitrile: 0.1% (v/v) aqueous acetic acid (11:16, v/v) at 1.0 ml/min. 20 µl of sample was added to the column and the concentration was calculated by using external standards.

Table 1 Ingredients of artificial diets with different contents of mulberry leaf powder

Group	Mulberry leaf powder (%)	Bean powder (%)	Maize powder (%)	Citric acid (%)	Gallic acid (%)	Sorbic acid (%)	Vitamin C (%)	VB mixture (%) ^a	Inorganic salt mixture (%) ^b
1	0	20.0	74.4	2.5	0.5	0.3	1.0	0.3	1.0
2	10.0	20.0	64.4	2.5	0.5	0.3	1.0	0.3	1.0
3	20.0	20.0	54.4	2.5	0.5	0.3	1.0	0.3	1.0
4	30.0	20.0	44.4	2.5	0.5	0.3	1.0	0.3	1.0
5	40.0	20.0	34.4	2.5	0.5	0.3	1.0	0.3	1.0
6	50.0	20.0	24.4	2.5	0.5	0.3	1.0	0.3	1.0

^aVB mixture consists of cyclohexanhexol, becholine, calcium pantothenate, niacin, benadon, riboflavin, aneurin hydrochloride, bioepiderm, and acidum folicum; ^bInorganic salt mixture contains K₂HPO₄·3H₂O, CaCO₃, KCl, MgSO₄·7H₂O, and FeSO₄·7H₂O

2.3 Data analysis

All the data were treated by statistical analysis applying to SAS 9.0 DA software. The absorption and enrichment of DNJ in silkworm larvae were calculated by the following formulas: $R_a (\%) = M_1/M_2 \times 100$, where R_a is the absorption ratio of DNJ during 1st to 4th instar or every day of the 5th instar, M_1 is the increased DNJ amount in body at every instar or every day of the 5th instar, and M_2 is the DNJ amount ingested at that instar or that day of the 5th instar; $R_e (\%) = M_3/M_4 \times 100$, where R_e is the accumulated enrichment ratio of DNJ by larvae, M_3 is the DNJ amount in larvae, and M_4 is the accumulated DNJ amount ingested.

3 Results

3.1 Relationship between DNJ contents in diets and in larvae

The silkworm larvae were fed with artificial diets varying with different contents of mulberry leaf powder or fresh mulberry leaves. The DNJ contents of artificial diets and the larvae of the 3rd day in the 5th instar were measured, and are presented in Table 2. The chromatograms of silkworm DNJ are shown in Fig. 1. The results show that the DNJ content in the artificial diets depended on the content of mulberry leaf powder. No DNJ could be detected in the diet

without mulberry powder, implying that mulberry powder was the sole source for DNJ. In the larvae, the DNJ content increased with the amount of DNJ in the diets. Statistical analysis demonstrated that there was a closely positive and linear relationship between the DNJ content in the diets (X) and the one in the larval body (Y) when euryphagous or general silkworm race was tested: $Y = 7.2111X + 0.0232$, $R^2 = 0.9154$ (Group 1, a euryphagous silkworm Guangshi); $Y = 5.2633X + 0.0129$, $R^2 = 0.9526$ (Group 2, general hybrid silkworm Guangshi×Fengyi). No DNJ was detected in the larvae raised with artificial diets without mulberry leaf powder, indicating that the larva could not synthesize DNJ by itself.

3.2 Variation of DNJ accumulation between male and female silkworm larvae

The results show that the DNJ content in male larvae was relatively higher than that in the females. There was a significant increase in DNJ content in both sexes when the larvae were fed with Nongsang 14 over Dajiguan and Husang 32 mulberry varieties (Table 3). The present results show that the male larvae could absorb and accumulate DNJ from diets more efficiently than females.

3.3 DNJ accumulation among silkworm varieties

The DNJ contents among various silkworm varieties were investigated (Fig. 2). The results show that there was a significant difference in DNJ content

Table 2 Effects of mulberry leaf powder on DNJ contents in artificial diets and silkworm

Mulberry leaf powder content in artificial diets (%)	DNJ content in artificial diets (%)	DNJ content in euryphagous silkworm (%) ^a	DNJ content in general hybrid silkworm (%) ^b
0	0	0	0
10	0.0148±0.0005	0.1862±0.0022	0.1244±0.0060
20	0.0333±0.0004	0.2062±0.0128	0.1670±0.0049
30	0.0363±0.0004	0.2984±0.0203	0.1906±0.0019
40	0.0439±0.0006	0.3228±0.0006	0.2380±0.0075
50	0.0482±0.0010	0.3982±0.0257	0.2863±0.0033
Fresh leaves	0.0918±0.0028	0.5706±0.0142	0.4608±0.0001

^a A euryphagous silkworm race, Guangshi, was used; ^b General hybrid silkworm race was used, commercial name: Guangshi×Fengyi

Table 3 Variation of DNJ contents between the female and male larvae^{*}

Morus species	DNJ content in mulberry leaf powder (%)	DNJ content in larval powder (%)	
		Female	Male
Nongsang 14	0.121±0.025	0.371±0.007	0.465±0.018
Dajiguan	0.116±0.010	0.286±0.032	0.341±0.006
Husang 32	0.107±0.060	0.257±0.010	0.316±0.036

^{*} Silkworm variety: Chunlei×Zhenzhu. The larvae of 3rd day in 5th instar were tested

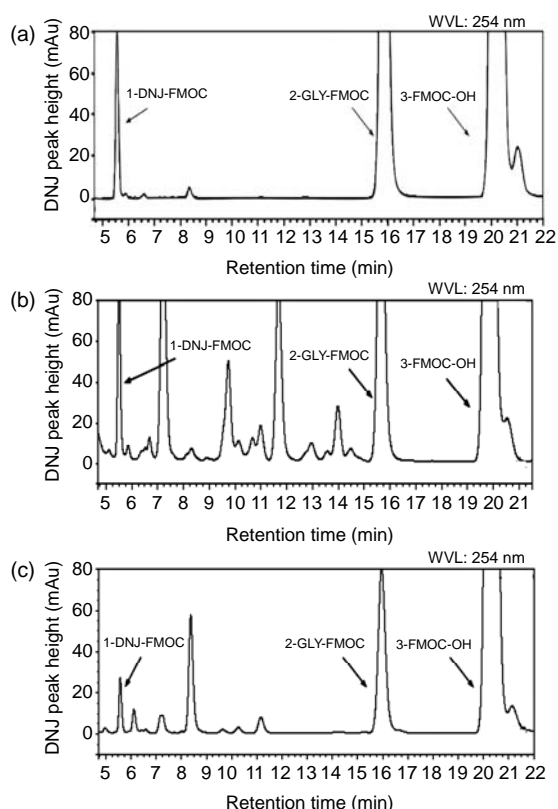


Fig. 1 High-performance liquid chromatograms of DNJ analysis

(a) Chromatogram of separation of DNJ standard solution; (b) Chromatogram of separation of mulberry powder solution; (c) Chromatogram of separation of silkworm powder solution. 1-GLY-FMOC: 1-glycine-fluorenyl-methyl chloroformate

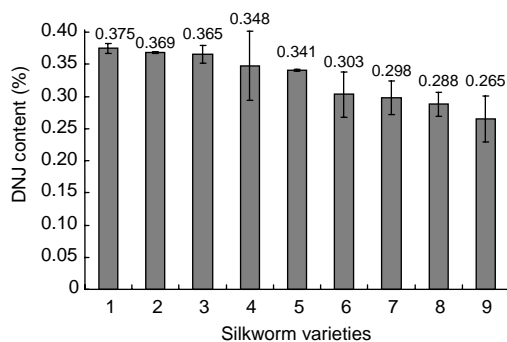


Fig. 2 DNJ contents among silkworm varieties

Silk worm varieties: 1, Jingsong; 2, Chunlei×Zhenzhu; 3, Luqi; 4, Haoyue; 5, 9202×Luqi; 6, 9202; 7, Luhuang; 8, Jingsong×Haoyue; 9, 973×974. DNJ was measured using the larvae of the 3rd day in the 5th instar. Each value is the mean±SE of replications

among various silkworm varieties. Among the races, the larvae of Jingsong showed a relatively high content of DNJ (0.375%), and a lower one was observed in hybrid silkworm 973×974.

3.4 Distribution of DNJ in larval organs or tissues

As silkworm variety Zhg×Chun 54 is the most popular and a commercial variety in production, this variety has better characteristics, such as a big body and being easy to raise for a large scale. Considering a medical use in the future, we chose this variety for the current study, except specially indicated. The distribution of DNJ in the larval body was investigated, and the results (Fig. 3) show that, except the silk gland, all organs and tissues contained DNJ. Among the tissues, there was a significant increase in DNJ content in blood, followed by intestinal juice and alimentary canal.

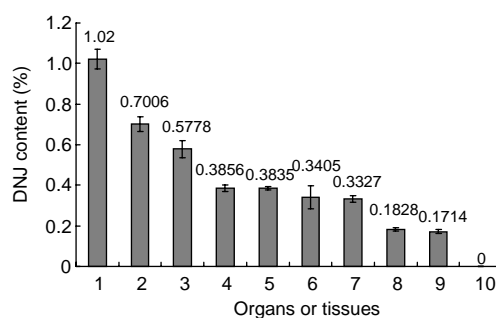


Fig. 3 DNJ distribution in larval organs and tissues

DNJ was measured using the larvae of the 3rd day in the 5th instar. Larval organs or tissues: 1, blood; 2, intestinal juice; 3, alimentary canal; 4, Malpighian tubule; 5, midgut peritrophic membrane; 6, cuticles; 7, body wall; 8, fat body; 9, trachea; 10, silk gland

3.5 DNJ content in mulberry leaf during growing stage

The DNJ contents in mulberry leaves of various growing stages were analyzed and are present in Fig. 4. The DNJ content was relatively high in tender leaves and decreased as the leaves matured into the medium-sized and coarse ones.

3.6 Accumulation and excretion of DNJ during larval stages

The results showed that the newly-hatched silkworm contained no DNJ, and then the DNJ content gradually increased until the middle of every instar, and then gradually decreased. The DNJ contents in the molting larvae were relatively low than that in the feeding larvae. The DNJ contents were sharply decreased from the 3rd or 4th day of the 5th instar, and no DNJ could be detected in the mature larvae.

DNJ content in the larval feces changed significantly during the growth period (Fig. 5). A significant increase in the DNJ content was observed during the 4th instar in feces over the other instars. In the 5th instar, the DNJ contents were stable for the first three days, and increased in the following two days and reached the peak one day before maturing larvae.

Based on the above results, it was noticed that DNJ contents in the larvae and the feces changed periodically and there was a close relationship of DNJ contents between the larvae and feces. DNJ content increased in larvae during the early and middle stages of every instar, while decreased during later stages, i.e., molting and mature larval stages. The present results indicate that early and middle instars were the main stages for DNJ accumulation in larvae, while at later stages DNJ content was mostly excreted and increased in the feces.

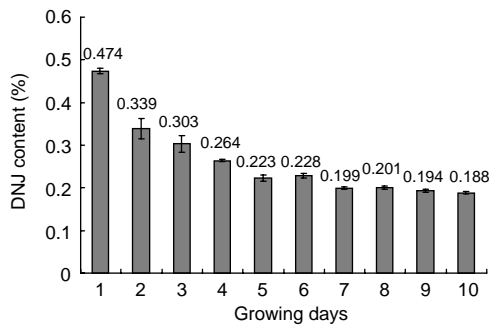


Fig. 4 DNJ contents in mulberry leaves during growing stages

Mulberry variety: Nongsang 14. Growing days refer the day order since the sprouting

The data of DNJ ingestion and accumulation in the larvae are presented in Table 4. The results show that the total DNJ content ingested during the whole larval stage was 9625 µg. DNJ accumulation in larval body reached the maximum on the 5th day of the 5th instar, and every larva contained 1825 µg of DNJ. From the 6th day of the 5th instar, no DNJ was observed in silkworm larvae; i.e., it was excreted. The absorption ratio on DNJ was higher on the 1st to 3rd days of the 5th instar, and the highest (44.41%) on the 2nd day. The accumulation ratio reached the peak on the 3rd day of the 5th instar by 33.80%.

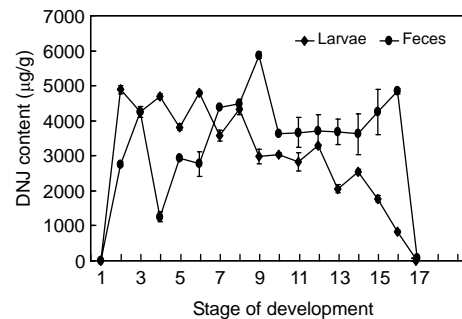


Fig. 5 DNJ change in the larval body and feces during the growing stages

Stage of development: 1, newly-hatched larva; 2, 3rd day of 1st instar; 3, molting stage of 1st instar; 4, larva in the middle of 2nd instar; 5, molting larva of 2nd instar; 6, larva in the middle of 3rd instar; 7, molting larva of 3rd instar; 8, larva in the middle of 4th instar; 9, molting larva of 4th instar; 10, 1st day of 5th instar; 11, 2nd day of 5th instar; 12, 3rd day of 5th instar; 13, 4th day of 5th instar; 14, 5th day of 5th instar; 15, 6th day of 5th instar; 16, 7th day of 5th instar; 17, mature larvae

Table 4 Enrichment of DNJ in larvae during different stages

Every instar and every day of the 5th instar	Ingested DNJ (µg/larva)	Cumulative ingested DNJ (µg/larva)	DNJ content in larva (µg/larva)	Increased DNJ in larva (µg/larva)	Absorption ratio (%)	Cumulative enrichment ratio (%)
Newly hatched			0.00	0.00		
1st instar	17.19	17.19	3.11	3.11	18.09	18.09
2nd instar	66.48	83.67	17.49	14.38	21.63	20.90
3rd instar	272.21	355.88	96.54	79.05	29.04	27.13
4th instar	1416.80	1772.68	379.14	282.60	19.95	21.39
Day 1, 5th instar	367.12	2139.80	537.54	158.40	43.15	25.12
Day 2, 5th instar	717.85	2857.65	856.37	318.83	44.41	29.97
Day 3, 5th instar	1279.99	4137.64	1398.65	542.28	42.36	33.80
Day 4, 5th instar	1211.22	5348.86	1643.24	244.59	20.19	30.72
Day 5, 5th instar	1365.08	6713.94	1825.46	182.22	13.35	27.19
Day 6, 5th instar	1392.66	8106.60	1815.85	-9.61	-0.69	22.40
Day 7, 5th instar	1060.13	9166.73	1037.01	-778.84	-73.47	11.31
Day 8, 5th instar	457.84	9624.57	631.74	-405.27	-88.52	6.56
Mature larva	0.00	9624.57	0.00	-631.74		0.00

4 Discussion and conclusion

There are many reports on the DNJ in *Morus*, and it was found that DNJ contents in mulberry leaves were affected by many factors, such as *Morus* species and seasons (Kimura *et al.*, 2007; Yatsunami *et al.*, 2008). However, almost no studies were done in the silkworm, and DNJ accumulation and excretion in the larva are not clear. This investigation showed that DNJ in the silkworm was ingested from diets, and its contents depended on the DNJ content in the diets, indicating that a silkworm larva could not synthesize DNJ by itself.

Our results indicate that the accumulation of DNJ in the larvae was affected by the sex, varieties, and developing stages of silkworms. The male larvae can accumulate DNJ more efficiently than the females. DNJ accumulation and excretion in the larvae were periodically changed. We conclude that it is the most effective and economic to develop DNJ-related products by using the male larvae of the 3rd day of the 5th instar, fed with leaves containing higher DNJ. In addition, the feces of silkworms before ecdysis and mature stage contain higher DNJ content and it can also be a resource for DNJ extraction.

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