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Free Fatty Acids Promote Feeding Behavior of the Silk-worm, *Bombyx mori* L.

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ABSTRACT It was found that adding one per cent of free fatty acids mixture of the mulberry leaves greatly increased feeding behavior of the newly hatched larvae. When separately tested with commercially obtained free fatty acids in accordance with each average value of six kinds of the confirmed ML free fatty acids, the experimental results showed that linolenic acid caused potent activity, then followed with linoleic acid, and lauric acid. Those of myristic acid, palmitic acid, stearic acid, and three octadecenoic acids (including elaidic acid, oleic acid, and vaccenic acid) caused no activity. Moreover, linoleic acid 0.09% plus linolenic acid 0.66% were found to have nearly same effectiveness as 1% ML free fatty acids mixture. And 1% of vaccenic acid also demonstrated promotive effect on larval feeding, but its activity was found lower than linoleic acid plus linolenic acid. Anyhow, those ML free fatty acids, especially linoleic acid plus linolenic acid, and vaccenic acid were proved having complemental efficacities with those ML-contained larvae's preferential substances such as sucrose and inositol. And those acids also acted synergestically with β-sitosterol, n-chlorogenic acid, and even vanillin to a fairly large extent under the existence of the biting assay diet.

Introduction

It was originally discovered by Hamamura (1) that the methanolic extract of the mulberry leaves (ML) caused high activity on the promotion of larval feeding. The biting factors was found present in both organic solvent soluble fraction (petroleum ether) and water soluble fraction of the above methanolic extract. When these two fractions were combined, and added to the methanol extracted residue of the ML, feeding behavior of the silk-worm larvae could be promoted to the utmost.

Talking about water soluble fraction, so far, some biting factors such as sucrose and inositol have been reported by Hamamura (2) (3). The monoglucoside pigment, isoquercitrin by Hayashiya (4), and n-chlorogenic acid by the authors (5) were also proved to increase feeding behavior of the newly hatched larvae. In concerning with organic solvent soluble fraction, up to now, one biting substance, β-sitosterol was previously described by Hamamura (3) from the non-saponified fraction, and linolenic acid by Wada *et al.* (unpublished). The free fatty acid fraction (2N-Na_2CO_3 extract), however, may be still containing more biting substances. Although linolenic acid by Ito *et al* (6). and oleic acid, the existence of which is not appreciable in ML, was reported by Yamada *et al* (7) that caused potent activities on the promotion of the growth, yet, on feeding stimulation, the particular contributions of these free fatty acids fraction to the silk-worm larvae have got to be clarified in details.

Therefore, in this paper, the divulged experiments of the commercial free fatty acids from C_{12} to C_{22}, and especially the ML-contained free fatty acids were carried out by using our special simplex biting assay diet (BAD) for the bioassay of their feeding stimulating efficacies.

Materials and Methods

Larvae F_1 of the strains Gunko x Manri, N1, N2, N3, of the Japanese strains, and S1, S2, S3, S4, S5 of the Chinese strains were used throughout the experiments. They were kindly provided by Gunze Silk Company.

Table 1. Components of the BAD

Components	g. of dry wt.
sucrose	0.1
inositol	0.02
cellulose	0.8
agar	0.2
buffer*	10 ml.

*0.05M (KOH + NaOH) was neutralized with M-H_3PO_4 to pH 6.8

Diet The BAD is shown in Table 1.

Method for check of feeding behavior The newly hatched larvae were placed on the BAD in a dark incubator at 27°C constantly. And then simply counted the number of the defecated feces at a certain time after fetching under non-sterile condition as previously described paper (5).

Free fatty acids Except self-prepared native ML free fatty acids mixture, ten kinds of free fatty acids were obtained commercially. The purities of these samples were examined by gas chromatography.

Preparation of free fatty acids The ML free fatty acids were prepared by the use of J. Bonner's method (8). Those commercially obtained six kinds of free fatty acids were combined according to the average values of six ML-contained free fatty acids from gas chromatograms as Table 2 shows. This mixture is designated as "6F" (8 kinds of ML components minus $C_{16:1}$ and $C_{18:1}$ acid. The former is omitted for its rarity and the latter for its difficulty in identification of its isomers). Another series of 7E, 7O, and 7V are the mixture of 6F plus elaidic acid, oleic acid, and vaccenic acid of each 0.92% separately. These three kinds of octadecenoic acids of each was added because they appeared

Table 2. Ratios of the ML free fatty acids compared with 7E, 7O, and 7V as calculated from gas chromatograms

Contents	Ratios (%)			Average values	7E	7O	7V
	Authors		Ito *et al.**				
	1st	2nd					
lauric acid	0.09	2.50	1.30	1.30	2.94	1.07	1.20
myristic acid	0.01	0.26	0.002	0.09	0.39	0.30	0.32
palmitic acid	23.58	24.00	13.90	20.50	26.46	28.10	29.00
$C_{16:1}$	0.09	0.09	0.65	0.28	0.05	0.12	0.09
stearic acid	2.54	0.70	1.40	1.54	0.07	0.85	0.65
octadecenoic acid	0.27	1.80	0.70	0.92	0.57	1.20	1.63
linoleic acid	9.60	4.65	13.00	9.08	0.85	1.50	2.43
linolenic acid	63.82	66.00	68.95	66.29	68.67	66.86	64.68

Note : The total amount of ML free fatty acids mixture is 100%. 7E, 7O, and 7V were combined according to the above 7 average values ($C_{16:1}$ was omitted).
* Those values were calculated by us from the gas chromatogram reported by Ito *et al.* (6).

sharply in the same position on the gas chromatogram as ML octadecenoic acid ($C_{18:1}$) appeared.

Here, we would like to point out that the commercially obtained free fatty acids were not so pure. For instance, from one Shimadzu gas chromatogram, we calculated that elaidic acid (cis-9-octadecenoic acid) was contaminated with 7.4% lauric acid, 5% myristic acid, 13% palmitic acid. Oleic acid (trans-9-octadecenoic acid) was found to have contaminated with 6.9% lauric acid, 8.2% myristic acid, 2.4% palmitic acid, and 7.2% $C_{16:1}$ unknown acid. And vaccenic acid (trans-11-octadecenoic acid) was also contaminated with 1.7% lauric acid, 3.4% palmitic acid, and 9.5% stearic acid.

The samples, 7E, 7O, and 7V, therefore, were analyzed to determine their compositions. Then we found palmitic acid greatly increased its quantity, and on the contrary, linoleic acid largely decreased. Since each industrial produced fatty acid was so impure, the true compositions of 6F were actually happened to be caused slight deflection, and hence more or less influenced the accuracy of our biological assays.

Results and Discussion

According to the quantitative analysis by means of gas chromatograph (Shimadzu GC-4B), eight peaks of the free fatty acids, extracted from the mulberry leaves powder, obtained clearly on the gas chromatograms, from which, the proportional value of each content was precisely calculated. Then we found the quantity of each fluctuation was quite different owing to the lots of material. For this reason, we decided to make an appropriate quantity from the calculated average value of each free fatty acid (see Table 2) for the bioassay of biting activity. Then it was found in this system that linolenic acid caused very high feeding efficacy than those of any other free fatty acids as Wada *et al.* previously discovered. Linoleic acid was lower than linolenic acid, while lauric acid also showed rather high activity. When each acid was added with β-sitosterol (0.5%) of the ML normal concentration reported by Hamamura (3), we found that lauric acid showed very high cooperative effect, then followed with linolenic acid, linoleic acid, and vaccenic acid. Those myristic acid, palmitic acid, stearic acid, and two octadecenoic acids (elaidic acid and oleic acid) though showed rather poor activities, yet, were still activated by it. The results are given in Table 3.

Table 3. The comparative effects of the commercially obtained free fatty acids on biting promotion (used BAD)

Contents	Addition (%)	Feces no. per 40 hrs per 10 larvae: without β-sitosterol	Feces no. per 40 hrs per 10 larvae: with β-sitosterol (0.5%)
lauric acid	0.013	146	316
myristic acid	0.0009	28	93
palmitic acid	0.20	11	54
$C_{16:1}$	—	—	—
stearic acid	0.015	2	64
octadecenoic acids :			
elaidic acid	0.009	7	27
oleic acid	0.009	0	11
vaccenic acid	0.009	55	108
linoleic acid	0.09	212	219
linolenic acid	0.66	406	464
control	—	0	73

Note : Each acid was dissolved in chloroform, and coated on the cellulose powder, then evaporated chloroform with electric water bath at 70°C.

Furthermore, when these free fatty acids were combined, we found the combination of linoleic acid plus linolenic acid gave 254 feces number for the first 18 hours (see Table 4). It demonstrated nearly equal effect as eight kinds of 1% ML free fatty acids mixture which was once reported by Yamada *et al.* (7) as a normal concentration in ML. It was also once analyzed by Ito *et al.* (6) as Table 2 shows. At the same experiment, 7V and 7E caused a little more higher activities than those of 7O and 6F, but lower than linoleic acid plus linolenic acid or natural ML free fatty acids mixture. Here we should remind that if linoleic acid was as pure as linolenic acid, we supposed that the promotive efficacies of these two fatty acids could be more increased.

On the other hand, when the assaying dosage of each acid was raised to 1%, it was found that vaccenic acid (trans-11-octadecenoic acid) showed fairly high specific function on feeding promotion. Elaidic acid was also proved to

Table 4. The effects of several kinds of free fatty acids mixture on biting stimulation (used BAD)

(a)

Contents	Addition (%)	Feces no. per 18 hrs. per 10 larvae
6F	ca. 1	196
7E	1	220
7O	1	153
7V	1	228
8F*	1	259
lauric + linoleic	0.013 + 0.09	6
lauric + linolenic	0.013 + 0.66	204
linoleic + linolenic	0.09 + 0.66	254
lauric + linoleic + linolenic	0.013 + 0.09 + 0.66	240

* Eight kinds of ML free fatty acids mixture

(b)

linoleic acid	0.09	36
linolenic acid	0.66	121
linoleic + linolenic	0.09 + 0.66	151
none	—	0

Table 5. The effects of the commercially obtained 10 kinds of free fatty acids on the promotion of larval feeding

Addition (1%)	Feces no. per 18 hrs. per 10 larvae	Remark
arachidic acid	82	These are one of six repeated experimental data. Each Petri dish was added with ML water soluble fraction 0.18g., cellulose 0.8g., agar 0.2g., and potassium phosphate buffer 10 ml. (pH 6.8)
erucic acid	58	
palmitic acid	33	
stearic acid	23	
elaidic acid	184	
oleic acid	34	
vaccenic acid	212	
linoleic acid	64	
linolenic acid	16	
ricinoleic acid	27	
8F	498	
none	96	

have activation next to vaccenic acid. Those of arachidic acid, erucic acid, linoleic acid, linolenic acid, oleic acid, palmitic acid, ricinoleic acid, and stearic acid were found to have caused inhibition on larval feeding. The resultant data are given in Table 5.

Since vaccenic acid was found having stimulation on feeding behavior, we were then tried to take a further step to test whether it acts synergestically with β-sitosterol by Hamamura (2), vanillin and n-chlorogenic acid by the authors (5). The experimental results showed that vaccenic acid surely cooperated with above biting substances to a large extent (see Table 6). In this

Table 6. The synergestical effect of vaccenic acid on the promotion of larval feeding under the existence of BAD

Petri dish No.	Sucrose (0.1g)	Inosite (0.02g)	n-chloro-genic acid (0.01g)	Vanillin (each 0.005g)	β-sito-sterol (each 0.005g)	Vaccenic acid (each 0.005g)	Feces no. per 20 hrs. per 10 larvae
1 A1* + C**	+	−	−	−	−	+	7
2 do.	+	−	−	−	−	−	0
3 do.	+	+	−	−	−	+	103
4 do.	+	+	−	−	−	−	35
5 do.	+	+	+	−	−	+	117
6 A2*** + C	+	+	+	−	−	+	225
7 A1 + C	+	+	+	−	−	−	70
8 do.	+	+	+	+	−	+	150
9 do.	+	+	+	+	−	−	131
10 do.	+	+	+	+	+	+	320
11 A2 + C	+	+	+	+	+	+	336
12 A1 + C	+	+	+	+	+	−	292
13 A1 + C	+	+	−	+	+	+	211
14 do.	+	+	−	+	+	−	134

Note :

* agar 0.2g
** cellulose 0.8g
*** agarose 0.2g

experiment, the comparative effects of the gelatinizers-agar and agarose were also studied, because the commercial agar contains considerable amounts of protein, oligosaccharides, natural pigments, ashes, and the influential effects of these impurities to the feeding behaviors of the silk-worm larvae can not be concluded. The results showed that agarose was actually a little more better than agar, and hence it was confirmed that these impurities in agar rather depressed biting activity.

Next step, in order to clarify whether unnatural 1% of vaccenic acid or natural 0.09% linoleic acid plus 0.66% linolenic acid activate feeding behavior highly, we were then used our BAD plus β-sitosterol plus n-chlorogenic acid, and even added vanillin to compare their activities. The summarized results showed that the promotive effects of linoleic acid plus linolenic acid further exceed artificial 1% of vaccenic acid, but at any rate lower than young ML as Table 7 shows.

Table 7. The comparison of the unnatural dose of vaccenic acid and natural doses of linoleic acid plus linolenic acid on feeding promotion (used BAD)

Components	Feces no. / 24 hrs. / 10 larvae
A*	258
A + 1 % vaccenic acid	285
A + 0.09% linoleic + 0.66% linolenic	427
A + 0.09% linoleic + 0.66% linolenic + 1 % vanillin	448
control (young ML)	461

* BAD + β-sitosterol 0.5% + n-chlorogenic acid 1%

Now, the feeding behavior of the newly hatched hybrid larvae of the F_1 of Gunko x Manri is clearly proved activated by free fatty acids. And what about the other strains? It is a well-known matter of fact that the Japanese strains are more active than Chinese strains to artificial diet in generally speaking, therefore, we particularly chose its parental strains : S1, S2, S3, S4, S5, and N1, N2, N3, and then used 7V plus BAD to examine their feeding behaviors. The experimental data of Table 8 clear-cut proved that three Japanese strains especially N1 and N3 were found really more active than all five Chinese strains, and even surpassed F_1 hybrid that has been using as larval material for

Table 8. The feeding behaviors of 8 kinds of strains compared to F_1 of Gunko x Manri (used BAD plus 1% of 7V)

Strains	Feces no. / 18 hrs. / 10 larvae
S1	15
S2	52
S3	17
S4	21
S5	28
N1	136
N2	58
N3	120
F_1 of Gunko x Manri	89

several years in our Kato Research Laboratory. Among Chinese strains, S1, S3, and S4 were found pretty inactive on feeding.

Further studies on the feeding preferential substances of the silk-worm larvae are still in progress.

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