STUDIES ON THIAMINE DISULFIDE

IV. MICROBIOLOGICAL ACTIVITIES OF O-BENZOYLTHIAMINE DISULFIDE DERIVATIVES

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For the microbioassay of thiamine, *Lactobacillus fermenti* 36 (1, 2) and *Lactobacillus viridescens* S38A (3) have been generally used. However, the activities of thiamine disulfide (TDS) derivatives on the growth of these organisms have not yet been reported except for the growth effect of TDS on *L. fermenti* (4—7).

The authors have performed the comparative studies on the growth effects of TDS and its derivatives, *e.g.* O-benzoylthiamine disulfide (BTDS), on these organisms under various conditions in order to clarify the vitamin activity of the above derivatives. It is possible that TDS and its derivatives show the vitamin activity after being converted to thiamine or its derivatives during sterilization or incubation period by the action of cysteine or cystine contained in the medium. Therefore, using the media containing varied amounts of cysteine or cystine besides the Macias-R and Deibel media, the activities of TDS and its derivatives in the case of autoclaving the derivatives with the basal medium were compared with those of aseptical addition of the derivatives to the sterilized medium.

EXPERIMENTAL

1. Samples

TDS and its derivatives used in the experiments were shown in Fig. 1. The quantities were expressed as the equivalents of thiamine hydrochloride (B_1 -HCl).

2. Test Organisms

L. fermenti 36 and *L. viridescens* S38A were used as the test organisms. As the stock culture, *L. fermenti* was incubated for 24 hours at 37° in the MaciasR medium (pH 5.5) (2) containing 1 μ g B_1 -HCl per ml and 1.5 % agar. This stab culture was stored at 5° and transferred weekly. *L. viridescens* was cultured in the Deibel medium (pH 6.8) (3), containing yeast extract untreated with alkali and 1.5 % agar, for 24 hours at 30°, and was stored and transferred in the same way.

3. Inoculum

Both the organisms were incubated for 17 hours at 37° or 30° in the liquid medium containing the same constituents as the stock medium except for agar.

FIG. 1 *Thiamine Disulfide and its Derivatives*

The cells were harvested, washed twice with 0.9 % sodium chloride solution, and the heavy inoculum (1:5) was used.

4. Sterilization

As shown in Fig. 2, the response curves of thiamine were the same after autoclaving for less than 5 minutes at 120° as those of aseptical addition. In the case of TDS derivatives the following two methods were used for comparing the activities.

(A) The sample was dissolved in sterilized water, and was added aseptically to the autoclaved basal medium.

(B) The sample was added to the basal medium and the mixture was autoclaved at 120° for 5 minutes.

FIG. 2 *Effect of the Sterilization of the Assay Medium on Responses of L. fermenti and L. viridescens to Thiamine Hydrochloride*

A: Thiamine-HCl solution was added aseptically to autoclaved basal medium.
B: The mixtures of thiamine-HCl solution and basal medium were autoclaved for 5 min (B-1), 10 min (B-2) and 20 min (B-3).
●, A, B-1; ×, B-2; ○, B-3.

5. Basal Media

As given in Table I—II, three media—MaciasR medium without cysteine (M-I), the medium containing cystine instead of cysteine (M-II) and the medium containing cysteine (M-III) — were used for the assay with *L. fermenti* 36. For the assay with *L. viridescens*, the three media given in Table II were used: the Deibel medium with some modification (D-I), the medium containing cystine (D-II) and the medium with cysteine (D-III). The modified Deibel medium contained KH_2PO_4 instead of K_2HPO_4 . In the original Deibel medium some precipitates were formed during sterilization, which did not disappear after standing for a long time. The modified medium also produced a little amount of precipitates by autoclaving, which

TABLE I
Basal Medium for L. fermenti

Component	Medium			Component	Medium		
	M-I	M-II	M-III		M-I	M-II	M-III
Casamino acids	g	g	g	Guanine	mg	mg	mg
Glucose	1.0	1.0	1.0	Uracil	2.0	2.0	2.0
	2.0	2.0	0.0		2.0	2.0	2.0
Xylose	0.4	0.4	0.4	Ca-Pantothenate	μg	μg	μg
Na citrate	1.0	1.0	1.0	Niacinamide	100	100	100
DL-Tryptophan	0.04	0.04	0.04	Riboflavin	100	100	100
L-Cysteine	—	—	0.1	PABA	200	200	200
L-Cystine	—	0.1	—	Pyridoxine-HCl	400	400	400
K_2HPO_4	0.5	0.5	0.5	Folic acid	20	20	20
CaCl_2	0.1	0.1	0.1	Biotin	2	2	2
					ml	ml	ml
NH_4Cl	0.2	0.2	0.2	Salts C ^a	2.0	2.0	2.0
	mg	mg	mg				
Adenine	2.0	2.0	2.0	Tween 80	0.2	0.2	0.2

The amounts given are for 100 ml double strength medium, pH 5.5.

^a Prepared according to Henderson and Snell.

TABLE II
Basal Medium for L. viridescens

Component	Medium		
	D-I	D-II	D-III
	g	g	g
Tryptone	2.0	2.0	2.0
Yeast extract	2.0	2.0	2.0
NaCl	1.0	1.0	1.0
Na citrate	1.0	1.0	1.0
KH_2PO_4	0.8	0.8	0.8
Glucose	2.0	2.0	2.0
L-Cysteine	—	—	0.1
L-Cystine	—	0.1	—
	ml	ml	ml
Salts C ^a	4.0	4.0	4.0
Tween 80	0.2	0.2	0.2

The amounts given for 100 ml double strength medium, pH 6.0.

^a Prepared according to Deibel *et al.*

disappeared after cooling, giving a clear solution.

6. Determination

Assay of the activity was carried out in a 15×120 mm uniform test tube and in a total volume of 2 ml. Incubation temperature was 37° for *L. fermenti* and 30° for *L. viridescens*. After incubation for 17 hours, turbidimetric estimation of growth was made directly in the tubes by use of the Hitachi EPO-B Type Electrophotometer at 660 mμ, and the results are expressed as optical density.

RESULTS

1. Effect of Sterilization on Growth Curves

It is desirable that the growth responses to thiamine remain unchanged regardless of the conditions of sterilization in order to compare the vitamin activities of TDS derivatives under various sterilization conditions. The activity of thiamine added aseptically to an autoclaved medium (A) was compared with the activities when thiamine was autoclaved for 5, 10 and 20 minutes respectively after being mixed with the basal medium (B). As shown in Fig. 2, the standard curves for thiamine of both *L. fermenti* and *L. viridescens* were the same after autoclaving at 120° for less than 5 minutes as those in which thiamine had been added aseptically.

2. Effects of Various Basal Media on Growth Curves

The growth responses of both the organisms in the basal media containing various amounts of cysteine or cystine—M-I, M-II, M-III and D-I, D-II, D-III—were compared. The results are given in Fig. 3.

FIG. 3 Growth Responses of *L. fermenti* and *L. viridescens* to Thiamine-HCl on Various Basal Media

The growth of *L. fermenti* in the MaciasR medium without cysteine (M-I) and that in the medium containing cystine instead of cysteine (M-II) were slightly poorer than that in the MaciasR medium (M-III), and the growth of *L. viridescens* was lowered when cysteine was added to the Deibel medium. However, these variation in the composition of the basal medium did not cause any marked effect on the thiamine activity to both the organisms.

3. Growth Responses to TDS and its Derivatives

The growth responses of both the organisms to TDS and its derivatives in the media containing various amounts of cysteine or cystine, sterilized in two different ways, are given in Figs. 4 and 5.

FIG. 4 Growth Response of *L. fermenti* to TDS and its Derivatives
Medium: M-I, M-II, M-III. A, B: method of sterilization

4. Activities of TDS and its Derivatives

The growth responses to 3 μg TDS and its derivatives per ml were compared turbidimetrically with that to thiamine at the same concentration, and the relative activities of TDS and its derivatives under various conditions were studied. As shown in Table III, the activities of TDS and thiamine disulfide cetylsulfate (TDS-CS) were less than that of thiamine when they were added aseptically to the sterilized M-I medium, although these derivatives showed the same activity with thiamine when autoclaved with the medium. In M-I or M-III medium, however, TDS and TDS-CS showed somewhat higher activities than thiamine after either steriliza-

FIG. 5 Growth Response of *L. viridescens* to TDS and Its Derivatives
Medium: D-I, D-II, D-III. A, B: method of sterilization

TABLE III
Activities of TDS-Derivatives to *L. fermenti*

Exp. No.	Cultural conditions		Relative activities (B ₁ -HCl=100)					
	Basal medium	Sterilization	TDS	TDS-CS	PTDS	ATDS	FTDS	BTDS
1	M-I	A	85	70	5	5	0	0
2		B	100	100	10	10	5	5
3	M-II	A	115	110	20	10	10	5
4		B	110	110	30	15	10	10
5	M-III	A	115	120	25	15	10	10
6		B	110	115	30	20	15	10

tion. BTDS, *O*-acetylthiamine disulfide (ATDS), *O*-propionylthiamine disulfide (PTDS) and *O*-furoylthiamine disulfide (FTDS) showed scarcely any activity in M-I medium, but they became somewhat active after autoclaving with cysteine or cystine. They showed about 10–30 per cent activity of thiamine when antoclaved with M-III medium.

TABLE IV
Activities of TDS-Derivatives to *L. viridescens*

Exp. No.	Cultural condition		Relative activity (B ₁ -HCl-100)					
	Basal medium	Sterilization	TDS	TDS-CS	PTDS	ATDS	FTDS	BTDS
1	D-I	A	75	80	20	10	10	0
2		B	110	115	70	45	35	10
3	D-II	A	110	110	75	35	40	10
4		B	120	115	80	40	45	10
5	D-III	A	115	110	80	35	45	10
6		B	120	110	80	35	45	15

On the other hand, as shown in Table IV, the activities of TDS, TDS-CS and BTDS to *L. viridescens* were almost the same as those to *L. fermenti*. The activities of ATDS, PTDS and FTDS in D-I medium without cysteine or cystine sterilized without autoclaving were found to be 10—20 per cent activity of thiamine. However, ATDS and FTDS showed 35—45 per cent of the activity and PTDS showed 70—80 per cent activity of thiamine in the medium sterilized by autoclaving or in that containing cysteine or cystine.

SUMMARY

The activities of thiamine disulfide (TDS) and its derivatives on the growth of *Lactobacillus fermenti* 36 and of *Lactobacillus viridescens* S38A were investigated under various conditions. The activities of TDS and its cetylsulfate (TDS-CS) to both the organisms in the medium without cysteine or cystine were lower than that of thiamine hydrochloride. Under the same conditions, *O*-propionylthiamine disulfide, *O*-acetyl-, *O*-furoyl- and *O*-benzoylthiamine disulfides showed only a slight activity. However, the activities of TDS and TDS-CS became higher than that of thiamine when the medium contained cysteine or cystine, especially the derivatives were autoclaved with the medium. The other derivatives showed 10—80 per cent activity of thiamine under the same condition.

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