ENZYMATIC REDUCTION OF SENNIDIN AND SENNOSIDE IN *PEPTOSTREPTOCOCCUS INTERMEDIUS*

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Peptostreptococcus intermedius, one of the dominant bacteria of human intestine, reduced sennidin and sennoside to rheinanthrone and 8-glucosyl-rheinanthrone, respectively, and these reduction rates were stimulated by the addition of nicotinamide adenine dinucleotide (reduced form) (NADH), flavin adenine dinucleotide (FAD) and glucose. The reduction was accelerated by removing oxygen from the incubation tubes, which indicates the inhibition of the reduction by O₂. Thus, for the maximal activity, the reaction system required an anaerobic condition and cofactors, NADH and FAD. This bacterium also reduced methyl orange, whose mode of reduction resembled that of sennidin reduction.

More than 80% of these activities were recovered in the supernatant fraction after sonication of the bacterial suspension, and these activities were purified together by means of Sephadex G-200 gel-filtration and diethylaminoethyl-cellulose column chromatography. The purified enzyme reduced FAD and benzyl viologen in the presence of NADH under an anaerobic condition.

Keywords — sennidin; sennoside; rheinanthrone; azo-dye; sennidin reductase; *Peptostreptococcus intermedius*

INTRODUCTION

Sennoside, the main constituent of *Sennae folium* or *Rhei rhizoma*, was considered to be inactive as a laxative itself but metabolized to active principles by intestinal bacteria in gut.^{1,2)}

From our investigations on sennoside metabolism by individual bacterial strains³⁾ and intestinal flora,⁴⁾ two different pathways for sennoside metabolism were proposed as follows; one was that sennoside was first reduced to 8-glucosylrheinanthrone, followed by hydrolysis to give rheinanthrone by β -glucosidase, and the other was that sennoside was first hydrolyzed to sennidin via sennidin-8-monoglucoside by β -glucosidase, followed by reduction to give rheinanthrone, which was a purgative principle.^{5,6)} Through these investigations, we discovered that several species of human intestinal bacteria reduced sennoside into 8-glucosylrheinanth-

rone,³⁾ and many species of bacteria reduced sennidin, aglycone of sennoside, into rheinanthrone.⁴⁾ Thus some of these bacteria were able to reduce both sennoside and sennidin.

In the present paper we report on the purification and properties of the reductase produced by *Peptostreptococcus intermedius*, because the bacterium is predominant in human intestine and has potent reducing activity for sennidin.

MATERIALS AND METHODS

Bacterium and Cultivation Conditions— *P. intermedius* from human feces was kindly provided from Prof. T. Mitsuoka of Tokyo University. The bacterium, maintained in General Anaerobic Medium (GAM) (Nissui) containing 0.15% agar, was first grown in GAM for 24 h at 37 °C in an anaerobic box, transferred to 10 volumes of the medium, and then cultured for 4 h at 37 °C

under an anaerobic condition.

Assay of Reduction Rates — Reduction of sennidin and sennoside into rheinanthrone and 8-glucosylrheinanthrone respectively was carried out as follows. The reaction mixture contained: 1 μmol of sennidin or 0.6 μmol of sennoside as a substrate; 2 μmol of nicotinamide adenine dinucleotide (reduced form) (NADH); 40 nmol of flavin adenine dinucleotide (FAD) and the enzyme in 0.5 ml of 50 mM K-phosphate buffer, pH 7.2. In order to examine the effect of various compounds on the reduction rate, NADH and/or FAD was removed or replaced by other cofactors. After incubation for 10–60 min at 37 °C under air or N_2 , the reaction was stopped by the addition of 0.2 ml of 1% *p*-nitroso-*N,N'*-dimethylaniline which was dissolved in pyridine. After being kept at room temperature for 10 min, 1.0 ml of ethylacetate in the case of sennidin or 1.0 ml of butanol in the case of sennoside was added, vigorously shaken, and then centrifuged at 3000 rpm for 5 min. A sample of the upper layer was applied to a polyamide thin layer plate, which was developed with pyridine-ammonia (100:3), in the case of sennidin, or pyridine-butanol-ammonia (50:50:3), in the case of sennoside. Azometin derivative of rheinanthrone or 8-glucosylrheinanthrone was quantitatively analyzed by a densitometer (Shimadzu CS-910) as described previously.⁴⁾

The reduction rate of an azo-dye such as methyl orange by a bacterial suspension was determined by measuring the time (min) required for the decoloration of the dye under air or N_2 . The reduction rate by a soluble enzyme was determined by measuring the absorbance at 500 nm under N_2 using a Thunberg-type cell. The reaction mixture contained 0.2 mM azo-dye, 4 mM NADH, 80 μM FAD and the enzyme in 0.5 or 2.0 ml of 50 mM K-phosphate buffer, pH 7.2. The mixture was incubated at 37 °C, in the case of the bacterial suspension or at 25 °C, in the case of the soluble enzyme.

Non-enzymatic rate of reduction of sennidin, sennoside and methyl orange was also determined in the presence of NADH and FAD

under an anaerobic condition and was subtracted from the enzymatic rate of reduction.

NADH-dependent reductase activities were measured spectrophotometrically using FAD, flavin mononucleotide (FMN), benzyl viologen and methyl viologen as electron acceptors at 450, 445, 600 and 600 nm, respectively, under N_2 using a Thunberg-type cell. The mixture contained 4 μmol of NADH, a suitable amount of an acceptor and the enzyme in 2.0 ml of 50 mM K-phosphate buffer, pH 7.2.

NADH oxidation,⁷⁾ and NADH-ferricyanide,⁸⁾ NADH-cytochrome c,⁹⁾ NADH-3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2*H*-tetrazolium bromide (MTT)¹⁰⁾ reductase and diaphorase¹¹⁾ using 2,6-dichlorophenol-indophenol (DCIP) and menadione as electron acceptors with or without FAD were measured according to conventional methods reported previously.

Protein Determination — Protein concentrations were determined according to the method of Lowry *et al.*¹²⁾ using bovine serum albumin (BSA) as a standard.

Determination of Molecular Weight — The molecular weight of NADH-dependent reductase was estimated by Sephadex G-200 column chromatography according to the method of Andrews¹³⁾ using phenol red, cytochrome c, ovalbumin, BSA, fructose-1,6-bisphosphate aldolase and blue dextran as molecular weight markers.

Chemicals — Sennosides A and B were isolated and purified from a crude sennoside powder (Nippon Funmatsu Yakuhin Co. Ltd.) according to the method reported previously.³⁾ Sennidins A and B, and rheinanthrone were prepared by the methods of Stoll *et al.*^{14,15)} NADH and nicotinamide adenine dinucleotide phosphate (reduced form) (NADPH) were purchased from Oriental Yeast Co. Ltd.

RESULTS

Reduction of Sennidin A and Sennoside A by P. intermedius

Hattori *et al.*⁴⁾ reported that *P. intermedius* was able to convert sennidin A to rheinanthrone

during its cultivation in GAM medium containing sennidin A. The cells collected from the culture also reduced sennidin A to rheinanthrone in K-phosphate buffer, pH 7.2. This reduction by the bacterial cell suspension was enhanced by the addition of FAD, NADH or glucose under air as shown in Table I. The mixture of both NADH and FAD was shown to be most active. However, these activities were markedly decreased by shaking the reaction vessels.

Under N₂, the bacterium showed a potent activity to convert sennidin A to rheinanthrone even in the absence of NADH, FAD and glucose, which was almost equal to the maximal reduction rate under air. The reduction under N₂ was also stimulated, but not markedly by the addition of FAD, NADH or glucose, suggesting that

the bacterium under N₂ was generating electron donors such as NADH. The maximal activity was also obtained by the addition of both NADH and FAD. NADPH acted as a poor electron donor. Similar results were observed for the reduction of sennoside A to 8-glucosylrheinanthrone, although the values obtained were low in comparison with those with sennidin A.

These results suggested that the reductive reactions required NADH, which was replaced by glucose in the viable cells, as an electron donor and FAD as an electron carrier, and were inhibited by oxygen.

Reduction of Methyl Orange by P. intermedius

P. intermedius was capable of reducing azo-dyes such as methyl orange. The reduction of methyl orange by viable cells was very weak in

TABLE I. *Reduction of Sennidin A and Methyl Orange by P. intermedius*

Addition	Reduction rate (nmol/min · ml)			
	Sennidin A		Methyl orange	
	Air	N ₂	Air	N ₂
None	2	65	0	10
FAD (80 μM)	11	144	N.D. ^{a)}	42
NADH (4 mM)	33	90	5.6	14
NADH (4 mM) + FAD (80 μM)	69	332	53	104
Glucose (20 mM)	56	93	15	19

After harvesting bacteria which were cultured for 4.5 h, the cells were suspended in 0.1 M K-phosphate buffer, pH 7.2. The bacterial suspension contained 7.1 mg of protein per ml. a) Reduction rate was not quantitatively determined because the orange color did not disappear completely.

TABLE II. *Reduction of Sennidin A and Methyl Orange by the Supernatant Fraction*

Addition	Reduction rate (nmol/min · ml)			
	Sennidin A		Methyl orange	
	Air	N ₂	Air	N ₂
None	0	< 0.1	0	0
FAD (80 μM)	0	17	0	0
NADH (4 mM)	5	123	N.D. ^{a)}	13
NADH (4 mM) + FAD (80 μM)	34	369	77	250

The supernatant fraction containing 5.7 mg of protein per ml was used. Reduction rates of methyl orange were determined by measuring time (min) required for decoloration. a) Same as that described in Table I.

the presence of FAD alone, but strong reduction occurred in the presence of glucose or NADH. The reduction of methyl orange was markedly enhanced by the addition of both NADH and FAD under an atmosphere of air as shown in Table I. Under an anaerobic condition, the bacterium reduced methyl orange without any addition, and the reduction rate was stimulated 1.4 and 1.9-fold by the addition of NADH and glucose, respectively, and 4-fold by FAD. The addition of NADH and FAD gave maximal reduction activity.

FIG. 1. *Reduction of Methyl Orange by the Supernatant Fraction*

A reaction mixture in a Thunberg-type cell contained 400 nmol of methyl orange and 2.3 mg of protein (the supernatant fraction as an enzyme source) in 2.0 ml of 50 mM K-phosphate buffer, pH 7.2. Reduction rate was measured as described under Materials and Methods. Eight μ mol of NADH and 160 nmol of FAD were added at the point indicated with arrows.

The results obtained using the azo-dye was similar to those with sennidin A, suggesting that reduction of sennidin and methyl orange in *P. intermedius* may proceed by a similar mechanism. *Reduction of Sennidin A by a Soluble Enzyme Obtained from P. intermedius*

A bacterial cell suspension was sonicated and then centrifuged to obtain supernatant and precipitate fractions. More than 80% of the activities for sennidin A and methyl orange reduction measured in the presence of NADH and FAD under N_2 was recovered in the supernatant fraction.

In the presence of NADH and FAD little sennidin A was reduced by the supernatant fraction under an atmosphere of air. However, under N_2 , a considerable amount of sennidin A was reduced by the addition of NADH and this reduction was further stimulated 3-fold by the addition of both NADH and FAD even though FAD alone stimulated the reduction poorly (Table II). Glucose was less active for reduction by sonicated cell suspensions and its supernatant fractions than by viable cells (data not shown). Sennoside was also reduced more efficiently by

FIG. 2. *Sephadex G-200 Gel Filtration of Sennidin and Methyl Orange Reductases*

Experimental procedures are described in the text. Fractions of 3 ml were collected. NADH-sennidin reductase with (○) and without (●) FAD, and NADH-methyl orange reductase activity (×) with FAD were measured as described under Materials and Methods.

sonicated cells and the supernatant fractions than by viable cells, suggesting that the bacterial cell membrane is less permeable to sennoside than sennidin. Reduction of methyl orange by the supernatant fractions proceeded similarly to that of sennidin (Table II). NADPH or cysteine did little to replace NADH as an electron donor.

The reduction of methyl orange was monitored spectrophotometrically by measuring the absorption at 500 nm. As shown in Fig. 1, methyl orange was not reduced by the addition of NADH or FAD alone, but was immediately reduced by the addition of FAD into the tube containing NADH. The dye was reduced after a lag time when the order of addition of both cofactors was reversed. The dye was also reduced

after a lag time by the simultaneous addition of both cofactors. However, under an anaerobic condition, the reaction proceeded immediately after the addition of both cofactors. These observations suggested that the lag period was due to the time required to deplete the oxygen in the reaction vessel.

Purification of NADH-Dependent Sennidin Reductase

The supernatant fraction was concentrated to a small volume by ultrafiltration. The concentrated supernatant fraction was applied to a Sephadex G-200 column (2.2×78 cm), equilibrated with 20 mM K-phosphate buffer, pH 7.2, and eluted with the same buffer. Both FAD-dependent sennidin A and methyl orange reductase activities were found together in two separate peaks (Fig. 2). The molecular weight of the major peak is about 120 K. The minor peak was found in the void volume. FAD-indepen-

FIG. 3. *DEAE-Cellulose Column Chromatography of Sennidin and Methyl Orange Reductases*

The fractions containing sennidin and methyl orange reductase activities from Sephadex G-200 gel-filtration were applied to a DEAE-cellulose column (2×5 cm), equilibrated with 20 mM K-phosphate buffer, pH 7.2. The column was eluted with 5 ml of 50 mM K-phosphate buffer, pH 7.2, and then with a linear gradient to 0.5 M NaCl over 200 ml. Fractions of 3 ml were collected. NADH-sennidin reductase with (○) and without (●) FAD, NADH-methyl orange reductase activity (×) with FAD, and NADH-ferricyanide reductase activity (Δ) were measured as described under Materials and Methods. Clear separation of FAD-independent and dependent sennidin reductase activities was confirmed in other experiments.

FIG. 4. *Isoelectric Focusing Column Chromatography of Sennidin and Methyl Orange Reductases*

The fractions containing sennidin and methyl orange reductase activities with FAD from DEAE-cellulose column chromatography were concentrated by ultrafiltration and the concentrated material was applied to a column containing ampholine (LKB, pH 4–6). The column was electrophoresed at 700 V for 2 d. After electrophoresis, 2 ml of fractions were collected. NADH-sennidin reductase (○) and NADH-methyl orange reductase (×) activities with FAD were measured as described under Materials and Methods.

dent sennidin A reductase activity was eluted in slightly later fractions.

Fractions containing these activities were collected and purified by diethylaminoethyl (DEAE)-cellulose column chromatography (Fig. 3). FAD-dependent NADH-sennidin reductase activity was eluted in the same fractions as NADH-methyl orange reductase activity, but they were clearly separated from FAD-independent NADH-sennidin reductase activity. The former fractions including sennidin reductase activity reduced MTT, DCIP and menadione in the presence of NADH, and also sennoside A and cytochrome c in the presence of NADH and FAD. The latter fractions showed NADH-dependent ferricyanide, MTT, cytochrome c reductase activities without FAD, but did not reduce sennoside A and methyl orange with and without FAD. Through these two steps of purification, the specific activities of the enzyme for sennidin A and methyl orange were 470 and 440 nmol/min · mg of protein respectively which represents a 20-fold purification when compared to the crude extract.

The purified enzyme was then subjected to isoelectric focusing column chromatography (pH 4–6), which revealed a single peak containing FAD-dependent NADH-sennidin and

methyl orange reductase activities and also sennoside A reductase activity. The isoelectric point for the enzyme was 4.9 (Fig. 4).

These results clearly showed that one enzyme was capable of reducing sennidin A, sennoside A and methyl orange in the presence of NADH, as an electron donor and FAD, as an electron carrier.

Properties of NADH-Sennidin Reductase

NADH oxidation by this enzyme was stimulated by menadione, FAD, FMN, benzyl viologen and methyl viologen. Under N₂, the enzyme reduced FAD, FMN, benzyl viologen and methyl viologen (Table III). These reduction rates were almost parallel to the reduction rates of sennidin A and methyl orange in the presence of FAD, FMN, benzyl viologen or methyl viologen. The highest activity was obtained by the addition of benzyl viologen, followed by FAD, then FMN and finally methyl viologen. Sennidin B and sennoside A were also reduced similarly, but NADPH showed less activity for all reduction reactions.

DISCUSSION

P. intermedius, one of the dominant bacteria in human intestine, has the ability to reduce sennidin and sennoside to rheinanthrone and 8-

TABLE III. Reduction of Sennidin A, Sennidin B, Sennoside A and Methyl Orange by the Purified Enzyme

Addition	Reduction rate (nmol/min · ml)				
	None	Sennidin A	Sennidin B	Sennoside A	Methyl orange
None	—	0	—	—	0
FAD (80 μM)	170	150	160	200	190
FMN (80 μM)	80	120	110	—	80
Methyl viologen (8 mM)	N.D. ^{a)}	80	—	—	N.D. ^{a)}
Benzyl viologen (8 mM)	1490	1230	1420	1250	1600

The reaction mixture contained 4mM NADH, enzyme, none or one of the substrates indicated above and none or one of the additives listed under the first column. The enzyme, which was purified by Sephadex G-200 column chromatography and DEAE-cellulose column chromatography steps, contained 1.7 mg of protein per ml. a) Reduction rate was not quantitatively determined in case of low activities.

glucosylrheinanthrone respectively as described in our previous papers.^{3,4)} In the present study, we discovered that this bacterium reduced an azo-dye such as methyl orange (Table I). FAD-dependent sennidin, sennoside and methyl orange reductase activities from *P. intermedius* were purified together by using Sephadex G-200 column chromatography and DEAE-cellulose column chromatography and an enzyme preparation was obtained as a single peak (pI=4.9) on isoelectric focusing column chromatogram. For the reduction of sennidin, sennoside and methyl orange, this enzyme required NADH as an electron donor and FAD or benzyl viologen as an electron carrier. These reduction rates were compatible with those of FAD and benzyl viologen in the presence of NADH. Gingell and Walker¹⁶⁾ reported non-enzymic reduction of azo-dyes by reduced flavins which were generated under an anaerobic condition by the enzyme in the supernatant fraction of *Streptococcus faecalis*. It was also proposed that azo-dyes were converted easily to free radicals by the reduced form of flavin and

the free radicals disappeared by transferring electrons to oxygen.¹⁷⁾ In regard to sennidin, Lemli *et al.*¹⁸⁾ reported that an anthraquinone dimer such as sennidin formed free radicals easily and the distribution of electron was not identical in the two moieties so that the C(10)-C(10') bond of this compound appeared to be weakened. Accordingly, sennidin, sennoside and methyl orange appear to be activated nonenzymatically by the reduced form of FAD or benzyl viologen, which is enzymically generated from NADH. The C-C bond and N=N bond of these compounds are then cleaved reductively under an anaerobic condition.

P. intermedius reduces sennidin and sennoside rapidly without NADH and FAD under an anaerobic condition. These reductive reactions appeared to be stimulated by the reduced form of natural coenzymes such as FAD, which is generated by the enzymes in the bacterial cells. In fact, the treatment of the bacteria with trypsin or pronase remarkably decreased these reduction rates. Many species of intestinal bacteria are able to convert sennidin and sennoside to rheinan-

CHART 1. *Metabolic Pathway of Sennoside in Gut*

throne and 8-glucosylrheinanthrone.^{3,4,19} It is suggested that these bacteria have enzyme systems possessing the capacity to reduce FAD. Thus, intestinal bacteria are required for the reduction of sennidin and sennoside to purgative principles, which is in agreement with the fact that caecal extracts from germ-free rats did not convert sennidin to rheinanthrone.²⁰

From our previous studies with individual bacterial strains isolated from human intestine and with human intestinal flora, different metabolic pathways of sennoside in gut have been proposed.^{3,4} From the present study, two pathways for sennoside metabolism in gut are deduced as shown in Chart 1. Sennoside could be either reduced or hydrolyzed to 8-glucosylrheinanthrone or sennidin. The respective metabolites could be next hydrolyzed or reduced to rheinanthrone, a purgative principle, in the anaerobic gut. These reactions could be catalyzed by the following enzymes derived from intestinal bacteria; NADH dehydrogenase to supply electrons to sennidin and sennoside *via* FAD, and β -glucosidase to hydrolyzed sennoside, sennidin-8-monoglucoside, and 8-glucosylrheinanthrone.

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