Purification and Some Properties of β -Glucosidase from *Streptomyces* sp.[†]

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Received June 24, 1986

β -Glucosidases I, II, and III were isolated from the culture filtrate of a *Streptomyces* sp. by ammonium sulfate fractionation, hydroxylapatite column chromatography, filtration on Bio-Gel P-100, and DE-52 column chromatography. β -Glucosidase III had a single active band on disc-gel electrophoresis. Its optimum pH and temperature for activity were 6.0 and 60°C, respectively. The isoelectric point and molecular weight of the enzyme were pH 4.5 and 45,000, respectively. From an experiment using ¹⁴C-labeled glucose, gentiobiose seemed to be formed from laminaribiose as isomaltose is formed from maltose by fungal α -glucosidase. The enzyme showed transglucosylation and produced gentiobiose from β -gluco-disaccharides and 4-*O*- β -D-glucopyranosyl-D-mannopyranose (epicellobiose). The enzyme acted on phenolic β -D-glucosides to produce unknown transfer products.

β -Glucosidases (β -D-glucoside glucohydrolase, EC 3. 2. 1. 21) are widely distributed among bacteria, fungi, yeasts, plants, and animals. Numerous reports on β -glucosidase have been published, but only a few reports on crude enzyme from *Streptomyces* can be found in the literature.^{1,2)}

We have reported two types of enzyme systems from species of *Streptomyces*,^{3,4)} which were isolated from soil using curdlan as a carbon source and a substrate. One of them, *Streptomyces* sp. W19-1, secreted both β -1,3-glucanase and β -glucosidase, and the cooperative action of both enzymes resulted in the formation of gentiobiose from curdlan (β -1,3-glucan). The gentiobiose-forming mechanism in this system was identified in our earlier study.⁵⁾ The β -1,3-glucanase hydrolyzed curdlan to produce glucose and laminari-oligosaccharides, and the β -glucosidase formed gentiobiose by transglucosylation from the resultant laminari-oligosaccharides, especially laminaribiose.

In our previous paper,⁶⁾ we described the purification of the β -1,3-glucanase from

Streptomyces sp. W19-1 and reported some properties of the enzyme. However, there was no report on transglucosylation by *Streptomyces* β -glucosidase. This paper describes the purification of *Streptomyces* β -glucosidase and the properties of the enzyme, especially the mechanism of the transfer reaction and some phenomena of transfer on various substrates by the enzyme.

MATERIALS AND METHODS

Chemicals. *p*-Nitrophenyl β -D-glucoside (PNPG), cellobiose (Ce), and phenyl β -D-glucoside (PhG) were purchased from Nakarai Chemical Co. (Japan). Sophorose (So) was prepared from a partial acid hydrolysate of stevioside, which was purchased from Sanyō-Kokusaku Pulp Co., Ltd. Laminaribiose (La)³⁾ and gentiobiose (Ge)⁴⁾ were prepared from hydrolysates of curdlan by the crude enzymes of *Streptomyces* sp. K27-4 and *Streptomyces* sp. W19-1, respectively. Epicellobiose (GM)⁷⁾ was prepared from the hydrolysate of konjac glucomannan by the β -mannanase of *Streptomyces* sp. No. 17. Curdlan (β -1,3-glucan) and salicin were purchased from Wakō Pure Chemical Co. (Japan). ¹⁴C-Labeled glucose (50 μ Ci, 250 μ l) was purchased from Amersham Japan Ltd. (Japan). All other chemicals were obtained commercially

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and were of analytical grade.

Preparation of the crude enzyme. *Streptomyces* sp. W19-1 was used in the preparation of the crude enzyme by the procedure described in our previous papers.^{4,5)}

Enzyme assays. PNPG-hydrolyzing activity was assayed by spectrophotometric measurement of *p*-nitrophenol released from PNPG as described in our previous paper.⁵⁾ One unit of the β -glucosidase activity was defined as the amount of the enzyme that released 1 μ mol of *p*-nitrophenol per min under the optimum conditions.

Measurement of protein. The method of Lowry *et al.*⁸⁾ was used for assaying protein, using bovine serum albumin as a standard. The amounts of protein eluted from columns were estimated from the absorbance at 280 nm.

Enzyme purification. The culture filtrate from *Streptomyces* sp. W19-1 has both β -1,3-glucanase and β -glucosidase activities, which could be separated by hydroxylapatite column chromatography after ammonium sulfate fractionation.⁵⁾ The adsorbed fraction on the hydroxylapatite column had β -glucosidase, and the unadsorbed fraction had β -1,3-glucanase activity which could be purified, as described in our previous paper.⁶⁾

The β -glucosidase-rich fraction after the hydroxylapatite column chromatography was concentrated by ultrafiltration using a membrane of UK-10 (Tôyô Roshi Co., Japan), and was dialyzed against 0.02 M phosphate buffer solution (pH 6.0) containing 0.1 M NaCl. The dialyzate was put on a Bio-Gel P-100 column (40 $\times$ 900 mm) equilibrated with the same buffer solution. The resultant β -glucosidase fraction was dialyzed against 0.02 M phosphate buffer solution (pH 8.0). The dialyzate thus obtained was put on a DE-52 column (25 $\times$ 300 mm) equilibrated with 0.02 M phosphate buffer solution (pH 8.0). After the column was washed with 1 liter of the same buffer solution, it was eluted by a linear gradient (0 $\sim$ 0.2 M NaCl).

Gel electrophoresis. Polyacrylamide disc-gel electrophoresis was done at pH 8.3 and 3 mA per column by the method of Davis.⁹⁾ After electrophoresis, protein in one gel was fixed with 12.5% trichloroacetic acid for 30 min and stained with 0.05% Coomassie Brilliant Blue R-250 (Sigma Chemical Co., U.S.A.). The other gel was also stained for β -glucosidase activity by the analogous method¹⁰⁾ of Erickson and Steers.¹¹⁾ After electrophoresis, the gel was placed in a solution containing 20% ethanol and 0.025% 6-bromo-2-naphthyl β -D-glucopyranoside (Nakarai Chemical Co., Japan) for 2 hr. Then the gel was rinsed in distilled water and transferred to a freshly prepared 0.1% Diazo Blue B (Sigma Chemical Co., U.S.A.) solution. After 2 min, the gel was destained and stored in a solution containing 10% methanol and 7% acetic acid.

Sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis was done by the method of Weber and Osborn.¹²⁾

To discover the isoelectric point, isoelectric focusing (IEF) was done in both a polyacrylamide gel¹³⁾ and a column¹⁴⁾ using Servalyt (Serva Feinbiochemica, West Germany) as the carrier. When IEF was done in the gel, protein in one gel was fixed with 12.5% trichloroacetic acid for 30 min and stained with 0.05% Coomassie Brilliant Blue G-250 (Sigma Chemical Co., U.S.A.). To detect the activity, the other unfixed gel was cut into about 2-mm-wide slices, and each slice was immersed in 0.5 ml of McIlvaine buffer solution (pH 6.0) for 3 hr. Then 0.5 ml of 2 mM PNPG was added to the resultant mixture and incubated for 3 hr at 55°C.

Paper and thin-layer chromatographies. Paper chromatography was done on a Tôyô-Roshi No. 51 filter paper sheet with a solvent system of 1-butanol-pyridine-water (6:4:3, v/v), and sugars on the sheet were detected by the silver nitrate method.¹⁵⁾ Thin-layer chromatography was done on a Merck TLC plate of Silica Gel 60 with a solvent system of chloroform-methanol-water (90:65:15, v/v) for the development of neutral sugars, and with a solvent system of chloroform-methanol-water (30:20:1, v/v) for phenolic β -D-glucosides. After developing, the sugars on the plate were detected by heating at 110°C for 10 min after spraying with 50% sulfuric acid.

Experiment using ¹⁴C-labeled glucose. A reaction mixture contained 5 μ l of ¹⁴C-labeled glucose solution (50 μ Ci/250 μ l), 50 μ l of 1% laminaribiose solution and 35 μ l of McIlvaine buffer solution (pH 6.0) was put into a test tube at 55°C. The β -glucosidase III (10 μ l, 0.2 units/ml) was added to the mixture and then the mixture was incubated at the same temperature. After 30 min, the mixture was boiled to stop the enzyme reaction and a part of the mixture was used for thin-layer chromatography (using an aluminum sheet). After developing, the sheet was dried by air and an autoradiogram was prepared by placing the sheet in contact with Fuji X-ray film for one week, followed by developing the film.

RESULTS

I. Enzyme Purification

DE-52 column chromatography, as shown in Fig. 1, was a very valuable method for separating the β -glucosidase system of *Streptomyces* into its component parts, and three peaks were observed with enzymatic activity. The three were designated as β -glucosidases I, II, and III in the order of elution, and each enzyme was collected separately. Table I summarized the process of purification of the

TABLE I. SUMMARY OF PURIFICATION OF β -GLUCOSIDASE FROM *Streptomyces* sp.

Purification step	Protein (mg)	Activity (units)	Specific activity (units/mg)	Purification (-fold)	Yield (%)
Culture filtrate	2,870	399	0.14	1	100
80%-saturated $(\text{NH}_4)_2\text{SO}_4$ fractionation	1,110	322	0.29	2.1	80.7
Hydroxylapatite	118	263	2.23	15.9	65.9
Bio-Gel P-100	48.4	232	4.80	34.3	58.2
DE-52					
β -Glucosidase I	3.3	27.1	8.21	58.6	6.8
β -Glucosidase II	2.9	51.9	17.9	128	13.0
β -Glucosidase III	4.9	62.2	12.7	90.7	15.6

FIG. 1. DE-52 Column Chromatography of the β -Glucosidase from *Streptomyces* sp. W19-1.

○—○, absorbance at 280 nm; ●—●, β -glucosidase activity; —, concentration of NaCl.

β -glucosidases.

β -Glucosidase III had several some protein bands on polyacrylamide disc-gel electrophoresis (Fig. 2A), but a single active band on the gel by the azo-coupling method (Fig. 2B). β -Glucosidases I and II had several protein bands on polyacrylamide gel electrophoresis, and gave two and three active bands, respectively (data not shown). β -Glucosidase III gave a single band on SDS-polyacrylamide gel electrophoresis (Fig. 2C).

II. Properties of the purified β -glucosidase

Physicochemical properties. Three β -glucosidases from *Streptomyces* were detected upon isoelectric focusing of the fraction after Bio-Gel P-100 column chromatography. The isoelectric points of β -glucosidases I, II, and III were pH 5.4, pH 5.1, and pH 4.5, re-

FIG. 2. Polyacrylamide Disc-gel Electrophoresis of the Purified β -Glucosidase III.

A: The purified enzyme (about 50 μ g) was electrophoresed. B: After electrophoresis, the gel was azo-coupled as described in MATERIALS AND METHODS. The band shows the β -glucosidase activity.

C: The purified enzyme (about 50 μ g) treated with SDS at 100°C for 5 min was put on a 7.5% gel containing 0.1% SDS, and run at 6 mA per column.

spectively. The isoelectric point of pH 4.5 was identical with that of the purified β -glucosidase III (Fig. 3).

The molecular weight of the β -glucosidase III was estimated as 45,000 by SDS-gel electrophoresis. β -Glucosidases I, II, and III were eluted together corresponding to the molecular weight of 45,000 by gel permeation liquid chromatography (data not shown).

Effects of pH and temperature on the β -glucosidase III. β -Glucosidase III had the highest

FIG. 3. Isoelectric Focusing (Gel) of the Purified β -Glucosidase III.

The purified enzyme solution (about 50 μ g) was electrophoresed using carrier Servalyt (pH 4~6). After electrophoresis, protein and β -glucosidase activity were detected as described in MATERIALS AND METHODS. Solid bars represent the enzyme activity. The insert shows the electrophoretogram of the purified enzyme stained with Coomassie Brilliant Blue R-250. Circles represent the pH.

activity at pH 6.0 (Fig. 4A) and was stable from pH 5.5 to 9.5 (Fig. 4C). The optimum temperature for the enzyme reaction was about 60°C (Fig. 4B). The enzyme was stable up to 55°C (Fig. 4D).

These effects of pH and temperature on the enzyme activity and stability were similar to these of the crude enzyme.

Effects of various compounds on the β -glucosidase III. The effects of metal ions and some reagents on enzyme activity were tested. As shown in Table II, Hg^{2+} and *N*-bromosuccinimide were strong inhibitors. Iodoacetic acid also affected the enzyme activity.

Transfer of glucosyl residue to ^{14}C -labeled glucose. We reported, in our previous paper,⁵⁾ the formation of gentiobiose from laminaribiose by *Streptomyces* β -glucosidase. To study the mechanism of the transfer reaction by β -glucosidase III, the enzyme reaction was done with the addition of ^{14}C -labeled glucose into a laminaribiose solution, under the conditions described in MATERIALS AND METHODS. Some of the resulting reactant and authentic

FIG. 4. Effects of pH and Temperature on Activity and Stability of the β -Glucosidase III.

A: Effects of pH on β -glucosidase activity. The enzyme reaction was done at various pHs (4~10) and 55°C for 10 min.

B: Effects of temperature on β -glucosidase activity. The enzyme reaction was done at various temperatures from 35 to 75°C for 10 min at pH 6.0 (McIlvaine buffer).

C: Effects of pH on stability. The enzyme solution was kept at 30 min for 1 hr in various pHs (4~10), and the remaining activity was assayed after adjustment to pH 6.0.

D: Effects of temperature on stability. The enzyme solution was incubated at various temperatures from 35 to 75°C under pH 6.0 for 10 min, and the remaining activity was assayed.

○—○, the purified β -glucosidase III (McIlvaine buffer system); ●—●, the purified β -glucosidase III (Atkins-Pantin buffer system); $\triangle$ --- $\triangle$, the crude enzyme (McIlvaine buffer system).

samples were chromatographed by TLC at the same time. After developing, the sheet was dried by air and cut into two parts. One part was autoradiographed. Another part was sprayed with 50% sulfuric acid to detect authentic samples. As shown in 1 of Fig. 5-I, the autoradiogram showed that β -glucosidase III formed several kinds of ^{14}C -labeled transfer products located under ^{14}C -labeled glucose. ^{14}C -Labeled gentiobiose was formed corresponding to the spot of authentic gentiobiose.

The ^{14}C -labeled gentiobiose was extracted from TLC plate with distilled water, then reduced by sodium borohydride,¹⁶⁾ and hydrolyzed by 10% trifluoroacetic acid. As shown in c of Fig. 5-II, radioactive glucitol was

TABLE II. EFFECTS OF VARIOUS COMPOUNDS ON *Streptomyces* β -GLUCOSIDASE III

The enzyme solution containing a test compound (1×10^{-3} M) was preincubated at pH 6.0 and 30°C for 1 hr. After the preincubation, the remaining activity was measured by the method described in our previous paper.⁶⁾

Compound	Relative activity (%)
None	100
BaCl ₂	93
CaCl ₂	100
CdCl ₂	92
CoCl ₂	92
CuCl ₂	94
FeCl ₂	73
FeCl ₃	101
HgCl ₂	2
NiCl ₂	92
PbCl ₂	99
ZnCl ₂	97
MnCl ₂	98
Sodium dodecyl sulfate	85
<i>N</i> -Bromosuccinimide	2
EDTA	82
Iodoacetic acid	64
<i>p</i> -Chloromercuribenzoate (PCMB)	92
Diethylpyrocarbonate	99

detected on the autoradiogram corresponding to the spot of authentic glucitol on both the autoradiogram (b of Fig. 5-II) and the thin-layer chromatogram (g of Fig. 5-II). This indicated that the ¹⁴C-labeled gentiobiose had a reducing-end ¹⁴C-labeled-glucose residue in its structure.

Action of the β -glucosidase III on gluco-disaccharides. To 0.5 ml of 1.0% solution of each gluco-disaccharide, we added 0.5 ml of the β -glucosidase III solution (containing 0.1 units in McIlvaine buffer solution, pH 6.0). At times after the beginning of incubation at 55°C, some of the reaction mixture was withdrawn and heated at 100°C for 10 min to inactivate the enzyme, and then used for thin-layer chromatography. As shown in Fig. 6, the enzyme degraded sophorose (So), laminaribiose (La), cellobiose (Ce), and gentiobiose (Ge), and transfer products in addition to glucose were formed simultaneously during

FIG. 5. Formation of ¹⁴C-Labeled Gentiobiose and Detection of Radioactive End Glucose Residue of ¹⁴C-Labeled Gentiobiose.

(I)-1, autoradiogram of the reactant resulting from the β -glucosidase reaction of ¹⁴C-labeled glucose and laminaribiose; (I)-2 to 4, thin-layer chromatogram of authentic gentiobiose, laminaribiose, and glucose, respectively; (II)-a and -b, autoradiogram of authentic ¹⁴C-labeled glucose and ¹⁴C-labeled glucitol, respectively; (II)-c, autoradiogram of ¹⁴C-labeled gentiobiose isolated from the reactant; (II)-d, autoradiogram of hydrogenated product (¹⁴C-labeled gentiobiitol) of (II)-c; (II)-e, autoradiogram of acid-hydrolysis product (¹⁴C-labeled glucitol) of (II)-d; (II)-f to -i, thin-layer chromatogram of authentic glucose, glucitol, gentiobiose, and gentiobiitol, respectively.

the hydrolysis of the above sugars. The transfer products moved more slowly than substrates on the chromatogram. Their behavior on thin-layer chromatography, therefore, suggested that the enzyme catalyzed both hydrolysis and a transfer reaction. In addition, the enzyme did not hydrolyze α -linkage in maltose, isomaltose, and sucrose (data not shown).

Action of the β -glucosidase III on 4-O- β -D-glucopyranosyl-D-mannopyranose. Action of the β -glucosidase III on 4-O-D-glucopyranosyl-D-mannopyranose (epicellobiose, GM) was investigated under the same conditions as for Fig. 6. As shown in Fig. 7, the enzyme degraded GM to produce glucose, mannose, and a transfer product presumed to be gentiobiose from its *R_f* value. But the

FIG. 6. Thin-layer Chromatograms of β -Glucobioses Hydrolysates with the β -Glucosidase III.

So, sophorose; La, laminaribiose; Ce, cellobiose; Ge, gentiobiose.

degradation of GM was slow since some sugar remained even after 3 hr of reaction. On the other hand, the enzyme did not hydrolyze β -1,4-mannobiose, β -1,4-xylobiose, or lactose (data not shown).

Action of the β -glucosidase III on PNPG, PhG, and salicin. Action of the β -glucosidase III on some phenolic β -glucopyranosides was also investigated under the same conditions as for Fig. 6. As shown in Fig. 8, the enzyme degraded PNPG, PhG, and salicin slowly, and was observed on the chromatogram to form transfer products which increased with the progress of reaction. However, these phenolic β -D-glucosides were not very susceptible since the substrates remained even after 3 hr of reaction.

DISCUSSION

In our previous papers,^{3,4} we had described two types of enzyme system from several species belonging to *Streptomyces*. One enzyme system, such as a culture filtrate from *Streptomyces* sp. W19-1 *etc.*, contained β -glucosidase (transglucosidase),⁵ and degraded curdlan to produce glucose and gentiobiose by transglucosylation. The other enzyme system, such as *Streptomyces* sp. K27-4 *etc.*, did not have the β -glucosidase activity and hydrolyzed curdlan to glucose and laminaribiose. Therefore, there are two groups of *Strepto-*

FIG. 7. Paper Chromatogram of Hydrolysates of GM (4-*O*- β -D-Glucopyranosyl-D-Mannopyranose) by the β -Glucosidase III.

A, authentic mannose, glucose and gentiobiose from top to bottom.

FIG. 8. Thin-layer Chromatograms of the β -Glucosidase III Action on PNPG, PhG and Salicin.

A, authentic glucose and gentiobiose.

myces that one secretes the glucosidase, and the another does not.

β -Glucosidases (β -D-glucoside glucohydrolase, EC 3.2.1.21) are widely distributed, and many workers have been reported on glucosidases from various organisms, for example, fungi, yeasts, bacteria, and *Strepto-*

FIG. 9. Schematic Presentation of the Possible Mechanism for the Formation of Gentiobiose from Laminaribiose by *Streptomyces* β -Glucosidase III.

Step I, formation of the enzyme-substrate complex; Step II, hydrolysis of laminaribiose and formation of the glucosyl-enzyme intermediate; Step III, transglucosylation by the enzyme.

myces. No reports, however, have appeared on research on the purification and properties of *Streptomyces* β -glucosidase. In this paper, β -glucosidases I, II, and III were obtained from the culture filtrate of *Streptomyces* sp. W19-1. The courses of degradation of laminaribiose by glucosidases I and II showed a similar tendency to that of glucosidase III, and the final products were also almost the same among the three enzymes (data not shown). The molecular weights and enzymatic properties of three enzymes were similar to each other, but the isoelectric points were different. Therefore, these results suggest that the enzymes are likely to be isozymes.

The molecular weight (45,000) of β -glucosidase III is similar to the values of 47,000 (*Trichoderma*¹⁷), 48,000 (*Candida*¹⁸) and 50,000 (*Clostridium*¹⁹), but it is different from the values of 120,000 ~ 160,000 (*Alcaligenes*²⁰) and 325,000 ~ 335,000 (*Saccharomyces*²¹).

From the experiment using ¹⁴C-labeled glucose (Fig. 5), it was proved that gentiobiose was produced by the transfer of non-reducing-end glucosyl residues of laminaribiose to free glucose, since the OH group at C-6 of the glucose is the most favorable acceptor site for *Streptomyces* β -glucosidase III. We consider this mechanism to be as follows (Fig. 9):

- 1) A laminaribiose-enzyme complex is at first formed between laminaribiose and

enzyme (Step I).

- 2) The reducing-end glucose of laminaribiose is removed from the laminaribiose-enzyme complex by its hydrolytic action, but the non-reducing-end glucosyl residue is allowed to form a glucosyl-enzyme complex (Step II).

We are forced to conclude that the formation of the complex seems to be favorable for the production of gentiobiose. That is to say, the β -glucosidase III could not produce gentiobiose in the reaction system of glucose only, even at a higher concentration and on longer periods of incubation (previous paper⁵). This suggests that the glucosidase is not able to form the glucosyl-enzyme complex with free glucose, not having a strong affinity for the glucose.

- 3) The glucosyl residue of the glucosyl-enzyme complex is transferred to the OH group at C-6 of free glucose, and gentiobiose is produced via the formation of a gentiobiose-enzyme complex (Step III).

At step III, no data of ours as yet exists to confirm this hypothesis, but we propose that the complex is formed temporarily for the production of the transfer product, because no transfer to other sugars than glucosyl residues, for example, mannose, mannobiose, xylobiose,

lactose *etc.*, could be observed with this enzyme.

The gentiobiose-forming mechanism of *Streptomyces* β -glucosidase III seems similar to the isomaltose-forming mechanism of fungal α -glucosidase²²⁾ which produced isomaltose from maltose. We suggest that the OH group at C-6 of D-glucose is also a favorable acceptor site for the β -glucosidase III.

Streptomyces β -glucosidase III could transglucosylate and produced gentiobiose and some other transfer products (unidentified oligosaccharides) from β -gluco-disaccharides (Fig. 6), epicellobiose (Fig. 7), phenolic β -D-glucosides (Fig. 8) and laminarioligosaccharides (previous paper⁵⁾). The relative rates liberating glucose from various sugars by the glucosidase were in the order of La $\gg$ So $>$ Ge $>$ Ce $\gg$ GM, La $>$ G₃ (laminaritriose) $>$ G₄ (laminaritetraose) $>$ G₅ (laminari-pentaose) $>$ G₆ (laminarihexaose) and PNPG $>$ Salicin $>$ PhG. The relative rates of production of gentiobiose from the above sugars almost corresponded to this order. Moreover, the optimum conditions of pH and temperature for the liberation of glucose from laminaribiose by the glucosidase agreed with those for the production of gentiobiose. Therefore, this conditions seems to be favorable to the formation of a glucosyl-enzyme complex followed by a gentiobiose-enzyme complex. The above results suggest that most suitable substrates for transfer will be those under which the substrate is split at a very high rate, and the resulting transfer product is hydrolyzed at a very low rate.

Various β -glucosidases have been reported, and the enzyme can be classified into two groups. One is hydrolases such as those of *Aspergillus aculeatus*²³⁾ and *Talaromyces emersonii*²⁴⁾ and another is transglucosidases such as those of *Alcaligenes faecalis*²⁰⁾ and *Aspergillus niger*.²⁵⁾ The glucosidase III from *Streptomyces* sp. W19-1 is grouped among transglucodases because the enzyme catalyzed a transfer action even at low substrate concentrations.

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