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Isolation and Properties of Levanase from *Streptococcus salivarius* KTA-19

NOBUYOSHI TAKAHASHI,[1]* FUMIKO MIZUNO,[2] AND KENSHIRO TAKAMORI[2]

Department of Oral Bacteriology, Tokyo Medical and Dental University School of Dentistry, Yushima, Bunkyo-ku, Tokyo 113[1] and Department of Oral Microbiology, Showa University School of Dentistry, Hatanodai, Shinagawa-ku, Tokyo 142[2]

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Fructan-hydrolyzing enzyme from *Streptococcus salivarius* KTA-19 isolated from human dental plaque was investigated. The enzyme was purified by ammonium sulfate precipitation, acetone fractionation, and column chromatography on Bio-Gel and DEAE-cellulose. The purified enzyme showed a single band on sodium dodecyl sulfate-polyacrylamide gel electrophoresis and isoelectric focusing. Its molecular weight was 100,000 as determined by sodium dodecyl sulfate polyacrylamide gel electrophoresis. The enzyme exhibited an optimum pH of 6.5 and decreased its activity from pH 6.0 and especially below pH 5.5. The optimum temperature was 40 to 50°C, and enzyme activity was reduced by 90% at 55°C. Enzyme activity was markedly inhibited by Hg^{2+}, Ag^{+}, Cu^{2+}, and *p*-chloromercuribenzoate at a concentration of 10^{-3} M, but not by other metal ions or chemical effectors. Fructose was the only by-product of the enzyme action on levan. These results indicated that the levanase of *S. salivarius* KTA-19 is an exo-β-(2,6)-fructofuranosidase.

The presence of fructan in dental plaque has been demonstrated by several authors (19, 23, 24). Fructan in dental plaque is synthesized extracellularly by many oral bacteria such as *Streptococcus salivarius* (11, 12, 14, 16, 27, 31), *Streptococcus mutans* (3, 4, 5, 8, 11), *Actinomyces viscosus* (19, 20, 25, 26, 28, 29, 36, 39), and *Rothia dentocariosa* (21). However, the fructan produced in dental plaque is not homogeneous. *S. salivarius* and *A. viscosus* produced fructan of the levan-type with (2,6)-linked β-fructofuranoside residues, branched in the 1-position (6, 12, 16), and *S. mutans* produced fructan of the inulin-type with (2, 1)-linked β-fructofuranoside residues with some branching in the 6-position (2, 11). On the other hand, several studies have shown that fructans synthesized in dental plaque are degraded or metabolized by some of the dental plaque organisms (7, 15, 18, 23, 37, 41). Several streptococcal strains in human dental plaque have been reported to hydrolyze levan produced by *S. salivarius*, *S. mutans*, and *A. viscosus* (7, 23, 25, 37, 41). The latter strain degraded its own levan (25). However, there have been few reports on the characteristics of the fructan-hydrolyzing enzymes of bacteria. Avigad and Bauer (1) isolated and purified a levanase preparation from an *Arthrobacter* species derived from soil origin. A levan-hydrolyzing enzyme of *A. viscosus* was partially purified by Miller and Somers (25). The fructan-hydrolyzing microorganisms in dental plaque have been studied and it has been found that approximately 37% of the plaque bacteria degrade fructans, including levan and inulin (35). The active organisms were *S. salivarius*, *S. mutans*, *Streptococcus sanguis*, *A. viscosus*, *Actinomyces naeslundii*, and *Bacteroides oralis*. *S. salivarius* was the most active organism to degrade streptococcal levan and inulin. In this study, we purified and characterized a levan-hydrolyzing enzyme produced by *S. salivarius* isolated from human dental plaque.

MATERIALS AND METHODS

Microorganisms. *S. salivarius* KTA-19 was used for the isolation of the levan-hydrolyzing enzyme and *S. salivarius* KT-12 was used for the synthesis of levan. Both strains were isolated from human dental plaque in our laboratory, and identified according to *Bergey's Manual of Determinative Bacteriology, 8th Ed.* (9) and the description by Hardie and Marsh (17). Their characteristics were described in a previous report (35). They were maintained on mitis salivarius agar (Difco Laboratories, Detroit, Mich.) or in brain heart infusion broth (Difco) in a frozen state.

Preparation of levan and its oligosaccharides. Levan was prepared from the supernatant of a culture of *S. salivarius* KT-12 in a brain heart infusion broth supplemented with 5% sucrose by the method of Feingold and Gehatia (13). Deproteinization was carried out by the method of Sevag et al. (30). The levan solution was dialyzed against deionized water, lyophilized, and stored at −20°C. The levan thus prepared contained no

detectable protein when assayed by the method of Lowry et al. (22), and more than 90% of the preparation was composed of ketohexose when determined by the method of Dische (10). Fructose was the only free sugar detected by analyzing acid-hydrolyzed products of levan with thin-layer chromatography. Mono- and oligosaccharides were obtained from partially hydrolyzed levan with 0.01 M HCl at 70°C for 30 min, neutralized with 5% Na_2CO_3, desalted by passing through the Amberlite IRA-900 and IRC-50 (Rohm & Haas Co., Philadelphia, Pa.) columns, and lyophilized.

Preparation of fructan-hydrolyzing enzyme. *S. salivarius* KTA-19 was incubated in a brain heart infusion broth supplemented with 0.2% levan prepared from *S. salivarius* KT-12 for 48 h at 37°C. The culture was centrifuged at 7,000 × *g* for 30 min at 4°C. Solid ammonium sulfate was added to the supernatant to 60% saturation, and the precipitate was collected by centrifugation at 8,000 × *g* for 20 min at 4°C. The precipitate was dissolved in 30 ml of 20 mM phosphate buffer (PB), pH 6.5, and dialyzed against 4 liters of the same buffer for 48 h at 4°C; the buffer was changed daily. After removing the insoluble material, the solution was lyophilized. This preparation was used as the crude enzyme preparation. Chilled acetone (−20°C) was added to a concentration of 35% (vol/vol) to the crude enzyme solution dissolved in 50 mM PB, and the solution was kept at 4°C for 5 h. The precipitate was removed by centrifugation at 4,000 × *g* for 15 min, as it contained no enzyme activity. Chilled acetone was again added to a final concentration of 45%, and the mixture was kept at −10°C for 20 h. The precipitate obtained by centrifugation was washed with chilled acetone and dried in vacuo. The dried enzyme preparation was dissolved in 10 ml of 20 mM PB, pH 6.5, and was applied to a column (2.5 by 100 cm) of Bio-Gel A5m (Bio-Rad Laboratories, Richmond, Calif.) equilibrated with 20 mM PB, pH 6.5. Elution was carried out with the same buffer at a flow rate of 20 ml/h, and fractions of 7 ml each were collected. The active fractions were pooled and concentrated by ultrafiltration, using a PM-10 membrane (Amicon Corp., Lexington, Mass.). The concentrated enzyme solution was applied to a DEAE-cellulose (DE-52; Whatman Biochemical Ltd., Kent, England) column (1 by 25 cm) equilibrated with 20 mM PB, pH 6.5. The elution was performed with a linear gradient of NaCl (0 to 1.0 M) in the same buffer at a flow rate of 25 ml/h, and fractions of 5.0 ml each were collected. Active fractions of DEAE-cellulose column chromatography were pooled and concentrated in an Amicon pressure cell with a Diaflo PM-20 membrane. The concentrated preparation was then applied to a Bio-Gel Al.5m column (2.5 by 70 cm) equilibrated with 20 mM PB, pH 6.5, and eluted with the same buffer at a flow rate of 15 ml/h.

Enzyme assay. The standard assay for levan-hydrolyzing activity was performed by incubation of 0.5 ml of a suitably diluted enzyme solution with 0.5 ml of 0.2% levan in 20 mM PB, pH 6.5, at 37°C for 30 min. The reaction was terminated by heating in a boiling water bath for 3 min. Reducing sugars, calculated as fructose, were determined by the method of Somogyi (33). The reaction products contained no free glucose when assayed with glucose oxidase reagents. One unit of levanase activity was defined as the amount of enzyme that liberated 1μmol of reducing sugar, equivalent to fructose, per min under standard conditions. Hydrolysis of sucrose was determined by the method of Sund and Linder (34) by monitoring the release of reducing sugar after the incubation of the enzyme with sucrose at 37°C. Inulinase activity was determined by the method of Snyder and Phaff (32).

SDS-polyacrylamide gel electrophoresis. Gel electrophoresis was used to estimate the molecular weight of levanase and to determine the homogeneity of the enzyme preparation, according to the method of Weber and Osborn (40), with 7.5% polyacrylamide–2% sodium dodecyl sulfate (SDS) gels. The enzyme solution was previously treated at 100°C for 5 min with 1% (wt/vol) SDS and 1% (vol/vol) β-mercaptoethanol.

Isoelectric focusing. Isoelectric focusing was performed on an LKB Multiphor model 2117, using 2% Ampholine, pH 3.5 to 10, at 30 W of constant power at 10°C for 2 h. The anode solution consisted of 1 M H_3PO_4 and that of the cathode was 1 M NaOH. After the focusing, the gel was stained with Coomassie brilliant blue by the method of Vesterberg (38). The pH gradient was checked with a mixture of pI calibration marker proteins (Oriental Yeast Co., Osaka, Japan), which yielded five protein bands corresponding to pH 4.1 through 9.7. Identical results were also obtained with an LKB surface electrode.

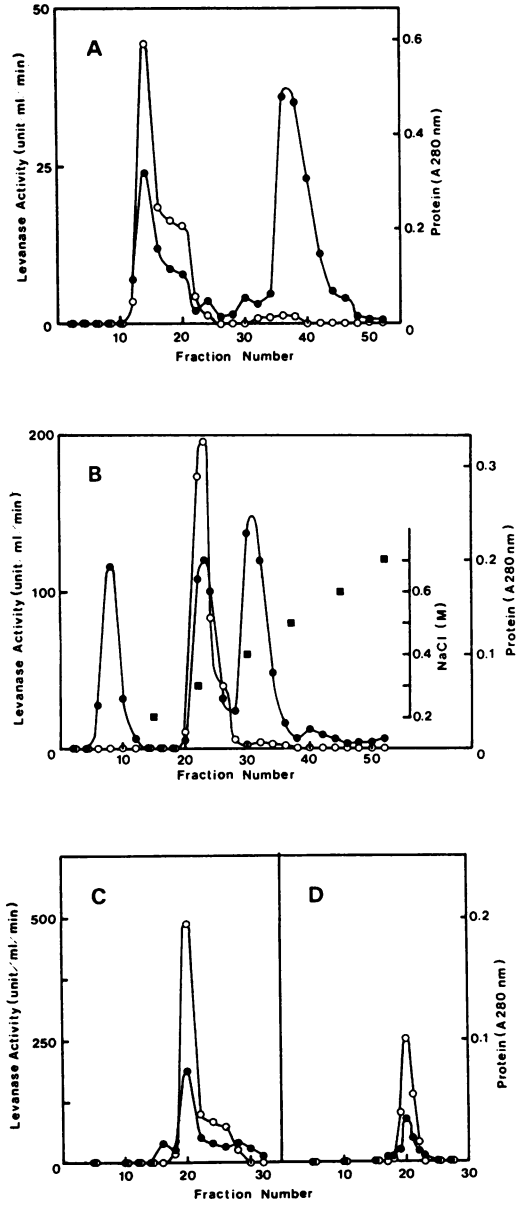
Thin-layer chromatography. Samples were applied to an Avicel SF (microcrystalline cellulose-acetate film) plate (Funakoshi Pharmaceutical Co. Ltd., Tokyo), dried, and subjected to development three times with a solvent of *n*-butanol/pyridine/water (6:4:4 in volume) at room temperature for 3 h. Sugar spots were visualized by spraying alkaline-silver nitrate solution on the plate.

RESULTS

Enzyme purification. When *S. salivarius* supernatant was precipitated by the addition of ammonium sulfate to 60% saturation and by acetone fractionation, approximately 80% of the original levanase activity was found in the sediment after centrifugation at 10,000 × *g* for 15 min. The results of the purification of levanase (using streptococcal levan as substrate) are shown in Table 1. The acetone precipitate was chromatographed on a Bio-Gel A5m column and the levanase activity was found in fractions 10 to 20 of the eluates (Fig. 1A). However, inulin- and sucrose-hydrolyzing activities were also detected in these fractions. Fractions from the Bio-Gel A5m column with over 2.0 U of levanase activity per ml were pooled, concentrated to 10 ml with an ultrafiltration apparatus, and applied to a DEAE-cellulose column. Levanase was eluted between 0.30 and 0.35 M NaCl by DEAE-cellulose chromatography (Fig. 1B). However, inulin- and sucrose-hydrolyzing enzymes still remained in the fraction. The fractions from a DEAE-cellulose column with levanase activity were pooled, concentrated by ultrafiltration, dialyzed, and applied to a Bio-Gel A1.5m column (Fig. 1C). In the final step of the purification, the enzyme was rechromatographed on the same Bio-Gel Al.5m column as a single symmetrical

TABLE 1. Summary of purification of levanase from *S. salivarius* KTA-19

Purification step	Total protein (mg)	Total activity (U)	Sp act (U/mg of protein)	Yield (%)	Purification (fold)
Crude enzyme	3,500	2,000	0.6	100	1.00
Ammonium sulfate precipitation	280	1,820	6.5	91.0	11
Acetone fractionation	227	1,635	7.2	81.8	12
Bio-Gel A5m	32	877	27.4	43.9	46
DEAE-cellulose	3.5	826	236.0	41.3	393
Bio-Gel Al.5m	0.5	405	810.0	20.3	1,350



peak which coincided with the only protein peak (Fig. 1D). The elution position of levanase in this chromatography corresponded to a molecular weight of 97,000 to 98,000. Twenty percent of the activity present in the initial extract was recovered throughout the purification procedure. Activity losses due to denaturation were minimal, and 80% of the activity was retained in the ammonium sulfate precipitation and acetone fractionation. This enzyme precipitation contained neither inulin- nor sucrose-hydrolyzing activities.

General properties of levanase. (i) Purity and molecular weight. The final enzyme preparation was homogeneous on SDS-polyacrylamide gel electrophoresis. The molecular weight of levanase was about 100,000 under denaturing conditions (Fig. 2), and that of the native form was about 97,000 to 98,000 as determined by gel filtration with Bio-Gel Al.5m.

(ii) Isoelectric point. The isoelectric point of the enzyme determined by thin-layer polyacrylamide gel electric focusing was pH 5.0 (Fig. 3).

FIG. 1. Chromatography of *S. salivarius* levanase. (A) Bio-Gel A5m chromatography. Acetone precipitate (45 mg), fractionated between 35 and 45% acetone, was applied to a Bio-Gel A5m column and eluted with 20 mM PB, pH 6.5. (B) DEAE-cellulose chromatography. Fractions with levanase activity eluted from a Bio-Gel A5m column (A) were pooled, concentrated, and applied to a DEAE-cellulose column and eluted with a 0 to 1.0 M NaCl gradient as described in the text. (C) Bio-Gel Al.5m chromatography. Fractions with levanase activity eluted from a DEAE-cellulose column (B) were pooled, concentrated, dialyzed, and subjected to gel filtration chromatography. Elution was carried out with 20 mM PB, pH 6.5. (D) Bio-Gel A1.5m chromatography. Fractions with levanase activity eluted from (C) were rechromatographed with the same buffer. Levanase activity, expressed as units per milliliter (○), was determined by using streptococcal levan as substrate as described in the text. Protein (●) was determined by absorbance at 280 nm. Salt concentration (M) (■) is expressed in terms of concentrations of standard NaCl solution.

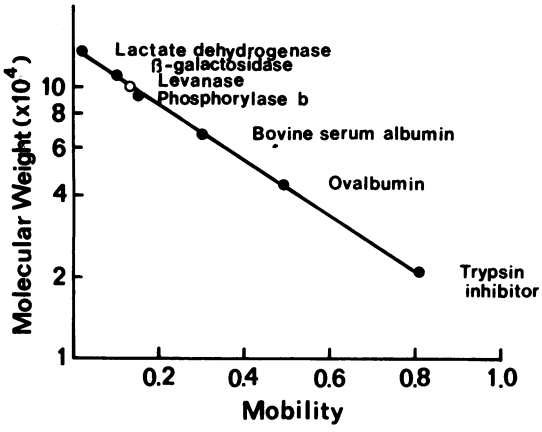


FIG. 2. Molecular weight determination of levanase by SDS-polyacrylamide gel electrophoresis. Molecular weights of the standard proteins were: lactate dehydrogenase, 140,000; β-galactosidase, 116,000; phosphorylase b, 94,000; bovine serum albumin, 67,000; ovalbumin, 43,000; and trypsin inhibitor, 20,100.

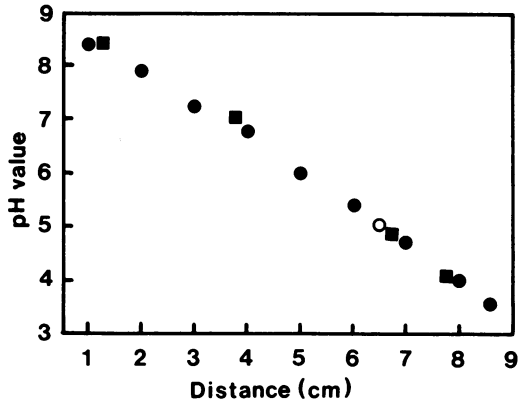


FIG. 3. Determination of isoelectric point of levanase from *S. salivarius* KTA-19. Isoelectric focusing of levanase was performed as described in the text; pH values were measured with pI calibration marker proteins (■) and with a surface electrode (●).

(iii) pH optimum. The effect of pH on the levanase activity was studied in the pH range of 3.0 to 8.0, using different buffer solutions: CH_3COOH-CH_3COONa buffer, pH 3.0 to 6.0, and Na_2HPO_4-NaH_2PO_4 buffer, pH 5.5 to 8.0. Enzyme activity was measured at 37°C with 5 μg of the enzyme. The optimal pH for enzyme activity was found to be at 6.5, and the activity decreased remarkably below pH 5.5 (Fig. 4).

(iv) Temperature optimum. Temperature dependence of the maximum reaction rate was studied in the range of 20 to 60°C. The optimum activity was obtained between 40 and 50°C, and no activity was detected at 55°C.

(v) Effect of metal ions and chemicals. The enzyme activity was not affected by Ca^{2+}, Mg^{2+}, Co^{2+}, and Zn^{2+} at a concentration of 10^{-3} M, but was markedly inhibited by Ag^{+}, Hg^{2+}, and Cu^{2+} (Table 2). The enzyme did not require any cations. It was found that the reaction was strongly inhibited by *p*-chloromercuribenzoate; it produced 71% inhibition of enzyme activity at a concentration of 10^{-6} M. In contrast, iodoacetate produced negligible inhibition at this concentration. The inhibitory effect of *p*-chloromercuribenzoate was found to be reversed by the addition of β-mercaptoethanol.

(vi) Substrate specificity. Under standard assay conditions, the enzyme was incubated with inulin, raffinose, sucrose, or melezitose, and the hydrolytic activity was compared with that of streptococcal levan. No activity was found for substrates other than levan.

(vii) Products of enzymatic hydrolysis of levan. Ten milliliters of the enzyme solution (5 U/ml) in 20 mM PB (pH 6.5) was incubated with an equal volume of 2% levan solution in PB at 37°C. Portions of the reaction mixtures were withdrawn at various intervals, and the amounts of reducing sugar released were determined. The enzyme completely hydrolyzed levan in 60 min under the above conditions. When the products

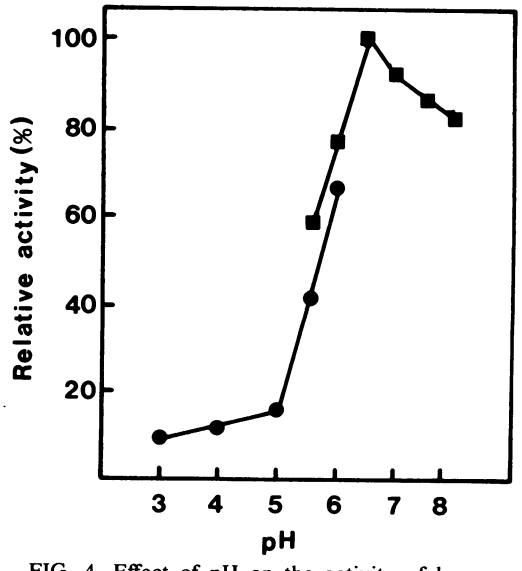


FIG. 4. Effect of pH on the activity of levanase from *S. salivarius* KTA-19. The enzyme activity at various pH values was assayed under the conditions described in the text. The maximum enzyme activity at pH 6.5 was taken as 100%. Acetate buffer was used for pH 3.0 to 6.0 (●), and PB was used for pH 5.5 to 8.0 (■).

of levan for various reaction intervals were determined, fructose was the only sugar detected on thin-layer chromatography. The amount of fructose released increased with reaction time (Fig. 5). This result showed that the mode of action of this levanase in the degradation of levan is an exo-type mechanism.

DISCUSSION

S. salivarius has been known to produce extracellular levan (11, 12, 14, 16, 27, 31), and many strains of streptococci in dental plaque degrade levan that they synthesize (7, 15, 18, 23, 41). However, few reports have been published on levan-hydrolyzing enzymes and their characteristics. A previous investigation demonstrated that *S. salivarius* strains in dental plaque are potent producers of fructan hydrolases. However, the whole cell culture degraded inulin as well as levan (35). In this experiment, fructan hydrolase was isolated from *S. salivarius* KTA-19 which had been isolated from human dental plaque in our laboratory (35). This streptococcal levanase cleaved only the β-(2,6)-fructofuranoside linkage of levan and did not hydrolyze the β-(2,1)-linkage of the inulin-type fructan. It also did not hydrolyze sucrose, raffinose, or melezitose containing 2-β-linkage. The pH optimum of 6.5 for this enzyme resembled that of *Arthrobacter* species reported by Avigad and Bauer. (1), as the only specific levan hydrolase of bacterial origin. However, the mode of action of this streptococcal levanase was different from that of *Arthrobacter*. Streptococcal levanase hydrolyzed levan and released only fructose. In contrast, *Arthrobacter* levanase cleaved levan at random and released a multitude of oligosaccharides containing various degrees of polymerization of about 2.5. The results indicated that streptococcal levanase was an exo-type and may be designated exo-β-(2,6)-fructofuranosidase. The activity of the enzyme was inhibited remarkably by Ag^+, Cu^{2+}, Hg^{2+}, and *p*-chloromercuribenzoate. Avigad and Bauer (1) reported that Tris was a noncompetitive inhibitor for *Arthrobacter* levanase, but it did not inhibit streptococcal levanase. In regards to oral bacteria, levan-hydrolyzing enzymes of *A. viscosus* were partially purified by Miller and Somers (25). The extracellular enzyme preparation from *A. viscosus* degraded its own levan and produced three carbohydrate spots on the thin-layer chromatography plate after a 3-h reaction, and free fructose increased after 8 h. The *A. viscosus* enzyme hydrolyzed inulin and sucrose as well as levan from various sources. The levan-hydrolyzing enzyme preparation of *A. viscosus* may contain more than one enzyme, since cell-associated and extracellular enzymes showed some differences in their modes of action. From our experience, the crude enzymes of *S. salivarius* showed similar action patterns.

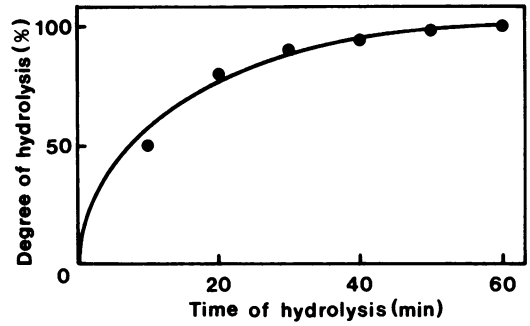


FIG. 5. Time course of levan hydrolysis by *S. salivarius* levanase. Purified enzyme was incubated with substrate in 20 mM PB, pH 6.5, at 37°C. Samples of reaction products were withdrawn at intervals, and the amount of reducing sugar was determined as described in the text.

TABLE 2. Effect of metal ions and chemicals on purified levanase[a]

Addition	Concn (m)	Relative activity (%)
Metal ions		
None		100
Ca^{2+}	10^{-3}	100
Mg^{2+}	10^{-3}	100
Co^{2+}	10^{-3}	100
Mn^{2+}	10^{-3}	100
Zn^{2+}	10^{-3}	100
Fe^{3+}	10^{-3}	113
Cu^{2+}	10^{-3}	19
Ag^{+}	10^{-3}	7
Hg^{2+}	10^{-5}	0
	10^{-6}	8
	10^{-7}	65
Chemicals		
None		100
KCN	10^{-3}	110
Disodium EDTA	10^{-3}	100
Iodoacetate	10^{-3}	100
Tris	10^{-3}	98
p-Chloromercuribenzoate	10^{-5}	0
	10^{-6}	29
	10^{-7}	76

[a] A mixture of 0.9 ml (5 U/ml) of the enzyme solution and 0.1 ml of each of the metal ions or chemicals at a concentration of 10^{-2} M was preincubated at 37°C for 30 min. After the preincubation, a levan solution in PB (pH 6.5) was added and incubated for 30 min.

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