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Purification and Properties of Hyaluronidase (EC 4. 2. 2. 1) from an Oral Strain of *Propionibacterium acnes*

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Summary

From a culture supernatant of *P. acnes* isolated from a lesion of periodontal disease, hyaluronidase was purified to homogeneity by the sequential procedures including ammonium sulfate precipitation, carboxy methy-cellulose column chromatography, and Sephadex G-100 gel filtration. Specific activity increased 1,027 fold and the recovery of the enzymatic activity was 12.4%. Molecular weight was determined to be 67,000 and isoelectric point was 7.2. Optimal pH for the activity was found at 5.5. The enzyme was inactivated by heating at 60°C for 10 min.

The purified hyaluronidase degraded hyaluronic acid, chondroitin, chondroitin sulfate A and chondroitin sulfate C. From the degradation products of these substrates, unsaturated disaccharides were detected by paper chromatography. When the rate of reaction of this enzyme against hyaluronic acid is supposed to be 100%, the corresponding values against chondroitin, chondroitin sulfate A, and C were 47%, 8%, and 8%, respectively. No degradation by this enzyme of heparin and heparan sulfate was demonstrated.

INTRODUCTION

Propionibacterium acnes is dominant normal inhabitant of the skin and mucous membrane of human adults[13]. It acts as an important etiological factor in local suppuration of the skin and the mucous membrane[9),24)]. *P. acnes* resides also in dental plaque and gingival crevice at level of 10^8 to 10^9 per one gram of the materials from these deposits[18)]. It was observed that this organism increased with the development of periodontal diseases[4)] and it was essential factor for the formation of experimental mixed infectious abcesses in animals by the oral indigenous bacteria[12),21)]. These findings suggest that *P. acnes* is a possible pathogen in oral flora.

It was revealed that *P. acnes* produced several extracellular enzymes including hyaluronidase[7),17),19)]. These enzymes have been regarded to be one of the etiological factors and thus, some of them have been purified and clarified their properties[3),8)]. However, only a little biochemical documents about the extracellular enzymes related to pathogenesis produced by oral *P. acnes* are available.

Nakamura et al. noted[14),15)] that a *P. acnes* strain isolated from a lesion of periodontal patient

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degraded acid mucopolysaccharides and lipids, but enzymatic analysis of these phenomena was not done. To understand the possible role of extracellular enzymes by this organism in periodontal disease, it is necessary to purify and characterize the enzymes which are thought to be responsible for pathogenicity of bacteria. In this report, we describe purification and biochemical properties of hyaluronidase from oral *P. acnes*.

MATERIALS AND METHODS

BACTERIAL STRAIN

P. acnes D-7 was isolated from a lesion of periodontal diseases[15]. For stock culture of D-7, the bacteria were anaerobically passaged every two weeks on agar slant containing brain heart infusion (3.7%), yeast extract (0.2%), and agar (1.5%).

PREPARATION OF AMMONIUM SULFATE FRACTION

D-7 was cultured in a liquid medium lacking agar from the above described medium anaerobically for 6 days. Ammonium sulfate was added to 70% saturation to the culture supernatant and the resultant precipitate was collected by centrifugation at 10,000 G for 20 min and dissolved in 0.05 M acetate buffer (pH 6.0), followed by dialysis overnight against the same buffer with several changes of the buffer.

SUBSTRATES

Hyaluronic acid (HA), chondroitin sulfate A (ChS-A),and chondroitin sulfate C (ChS-C) were purchased from Sigma Chem. Co. Chondroitin sulfate B (ChS-B), Chondroitin (Ch), and heparan sulfate (Hep-S) were obtained from Seikagaku Kogyo Ltd. Heparin (Hep) was purchased from Wako Pure Chemical Industries Ltd.

ENZYME ASSAY

According to Strominger et al[20]., hyaluronidase activity was assayed by appearance of color of *N*-acetylglucosamine end-group using umbilical cord hyaluronic acid as a substrate. 0.2 ml of enzyme samples diluted in 0.05 M acetate buffer (pH 6.0) were added to 0.8 ml of substrate solution (1.25mg/ml), previously been warmed at 37°C for 15min. The reaction mixtures were incubated at 37°C for 10 min. After incubation, 0.2 ml of 0.8 M borate buffer (pH 9.0) was added and heated for 3 min in boiling water to cease reaction, followed by chilling in ice water bath. After addition of 6 ml of *p*-aminobenzaldehyde reagent to these materials, they were incubated further at 37°C for 20 min and measured absorbance at 585 nm. One unit of enzymatic activity was defined as the amount of enzyme that released 1 μmole of *N*-acetylglucosmamine from HA per min under these conditions. The enzymatic activities against the other substrates were assayed by measurement of liberated Δ 4, 5-unsaturated disaccharides by absorbance at 232 nm[25].

ESTIMATION OF PROTEIN

Protein was assayed by the method of Lowry et al.,[11] using bovine serum albumin as a standard.

POLYACRYLAMIDE GEL ELECTROPHORESIS

Purity of the samples was examined according to Davis[5], using polyacrylamide gel electrophoresis. Concentration of gel was 7.5%.

ISOELECTRIC FOCUSING

To determine pI value, column (110 ml) isoelectric focusing (LKB) was employed[23]. Focusing was carried out for 24 h under constant voltage (300 V). The column was maintained at about 4°C with a circulating water bath.

DETERMINATION OF MOLECULAR WEIGHT

The molecular weight of hyaluronidase was determined by gel filtration on Sephadex G-100 column by the method of Andrews[2]. Standard proteins were bovine serum albumin, ovalbumin, chymotrypsinogen, and cytochrome C.

PAPER CHROMATOGRAPHY

To identify the sugars derived from the substrates by the action of the purified hyaluronidase, paper chromatography was carried out. Reaction mixtures of the enzyme and substrates were incubated for 4 to 8h, and the supernatant solution of these reaction mixtures obtained by centrifugation were spotted onto Tokyo filter paper No. 50A. Materials in the spots were developed descendingly using a solvent consisting of isobutyric acid and 1 M ammonia water in a volume ratio of 5 : 3[6]. Detection of sugars were carried out by color reaction with aniline hydrogen phosphate spray[16] and irradiation of ultraviolet light at 253.7 nm. Authentic unsaturated disaccharides and hyaluronidase (from *Streptomyces hyalurolyticus*) for standard were purchased from Seikagaku Kogyo Ltd.

RESULTS

PURIFICATION

As we revealed earlier that acid mucopolysaccharidase activity was found in culture supernatant of this organism[14),15)], the culture supernatant was used for the starting material for purification of hyaluronidase. To a culture supernatant (1,800 ml), ammonium sulfate was added at 70% saturation and the precipitate was collected by centrifugation at 10,000 G for 20 min. The precipitate was dissolved in 180 ml of 0.05 M acetate buffer (pH 6.0) and dialyzed against the same buffer. The ammonium sulfate fraction was then applied to a column (40 by 2.6 cm) of carboxy methyl (CM)-cellulose, previously been equilibrated with 0.05 M acetate buffer (pH 6.0) and the column was washed thoroughly with this buffer and eluted with linear gradient of NaCl in the buffer. In this chromatography, the majority of proteins in the sample were removed in the negative adsorption fraction and in the early eluates from the column with dilute NaCl. As illustrated in Fig.

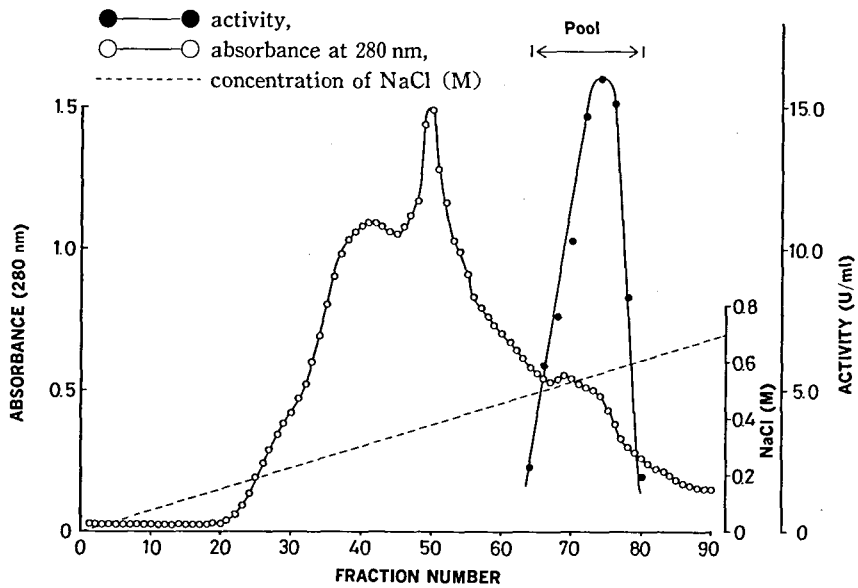

Fig. 1 : CM-cellulose column chromatography of hyaluronidase

1, the activity emerged as a single peak at NaCl concentration of 0.5 M to 0.6 M. The active fractions were combined (98 ml) and concentrated to a small volume *in vacuo* with a rotory evaporator at 30°C. The concentrated active fraction was dialyzed against 0.05 M phosphate buffer

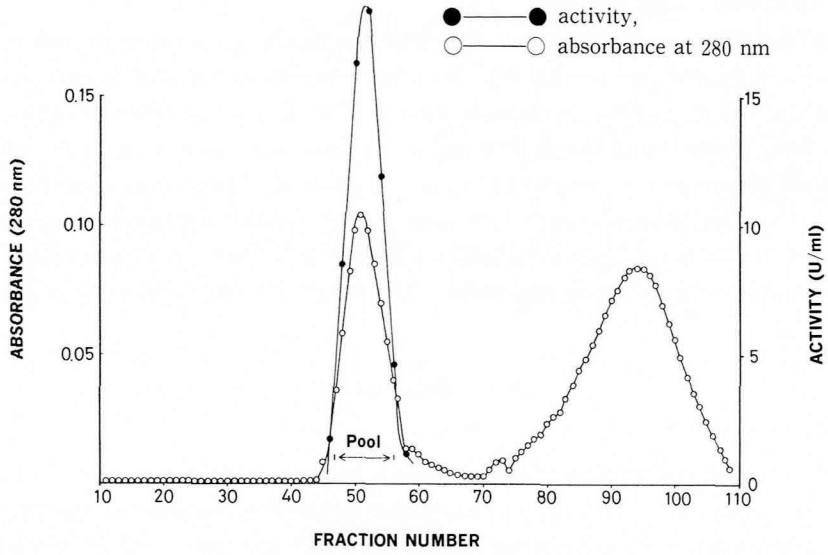

Fig. 2 : Gel filtration of hyaluronidase on Sephadex G-100 column

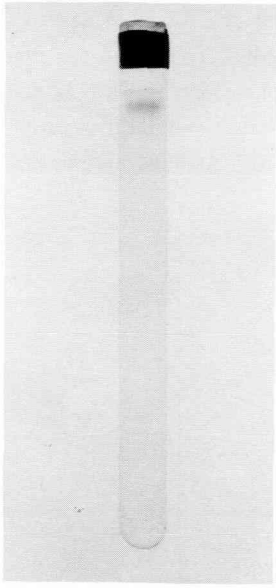

Fig. 3 : Polyacrylamide gel electrophoresis of the purified hyaluronidase. Migration is from top to bottom.

Table 1 : Purification of hyaluronidase

Step	Volume (ml)	Total protein (mg)	Total activity (U)	Specific activity (U)	Purification (fold)	Yield (%)
Culture supernatant	1,800	25,740	4,230	0.16	1	100
70% Saturate ammonium sulfate precipitate	180	5,670	2,925	0.52	3	69.1
CM-cellulose (CM-32)	98	43.1	1,068	24.88	156	25.2
Sephadex G-100	40	3.2	526	164.38	1,027	12.4

Table 2 : Effects of metal ions on the activity of hyaluronidase

Ions (10^{-3} M)	Percent of activity
None	100
Ca^{2+}	132
Mg^{2+}	147
Mn^{2+}	134
Fe^{2+}	78
Cu^{2+}	0
Hg^{2+}	0

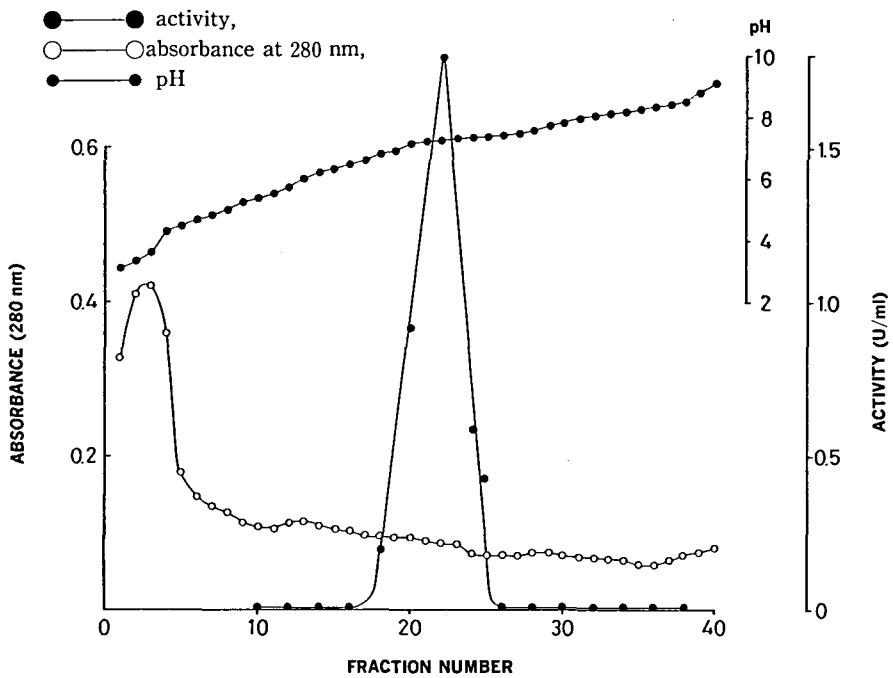

Fig. 4 : Isoelectric focusing of the purified hyaluronidase

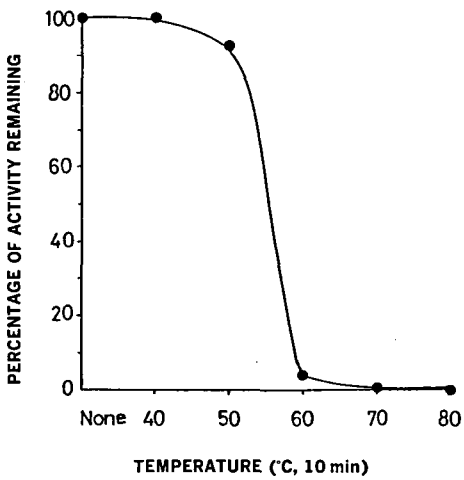

Fig. 5 : Thermostability of hyaluronidase

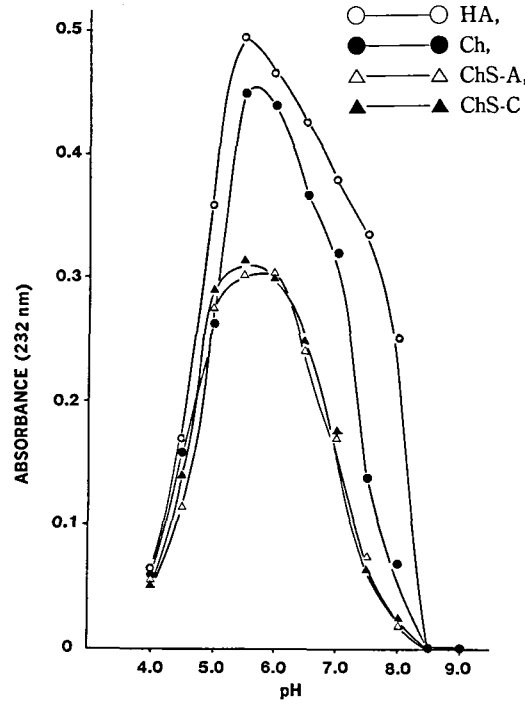

Fig. 6 : Effect of pH on the activity of hyaluronidase

(pH 7.0) containing 0.15 M NaCl and subjected to gel filtration on Sephadex G-100 column (90 by 2.6 cm) and eluted with the same buffered saline. Elution profile (Fig. 2) shows that two main protein fractions were separated and the activity was found at the position corresponding to the protein fraction eluted earlier.The active fractions (40 ml) were combined and concentrated similarly and finaly dialyzed against 0.05 M phosphate buffer (pH 7.0).

Fig. 3 shows polyacrylamide gel electrophoresis of the purified hyaluronidase. A single stained protein band was observed.

Table 1 summarizes purification of hyaluronidase. The specific activity increased 1,027 fold. The recovery of the activity was 12.4%

ISOELECTRIC POINT

Isoelectric point was determined to be 7.2, using isoelectric focusing (Fig. 4).

MOLECULAR WEIGHT

From the plot of elution volumes and molecular weights in Sephadex G-100 gel filtration, the molecular weight of hyaluronidase was estimated to be 67,000.

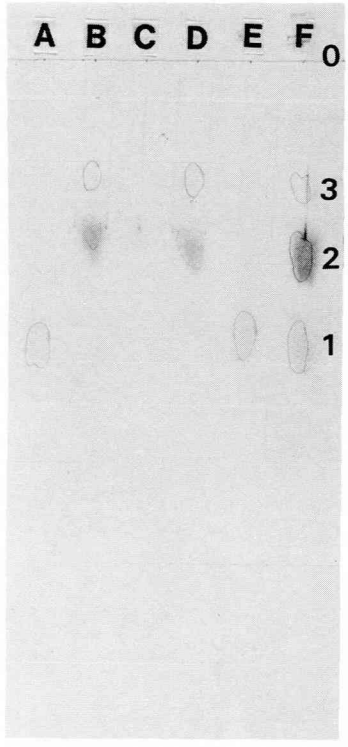

Fig. 7 : Paper chromatographic separation of disaccharides in degradation products of HA, ChS-A, ChS-B, ChS-C, and Ch by hyaluronidase.
A ; HA digest, B ; ChS-A digest, C ; ChS-B digest, D ; ChS-C digest, E ; Ch digest, F ; authentic unsaturated disaccharaides, 1 ; △ Di-0S, 2 ; △ Di-4S, 3 ; △ Di-6S O ; origin.

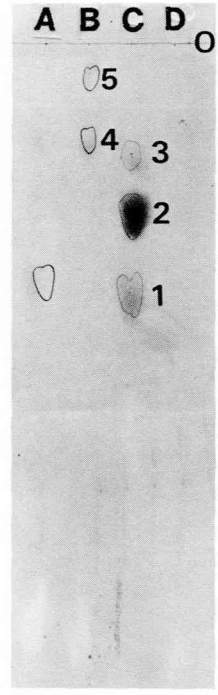

Fig. 8 : Paper chromatograms of degradation products in HA by *p. acnes* hyaluronidase and *Streptomyces hyalurolyticus* hyaluronidase.
A ; *P. acnes* hyaluronidase, B ; *S. hyalurolyticus* hyaluronidase, C ; authentic unsaturated disaccharides, 1 ; △ Di-0S, 2 ; △ Di-4S, 3 ; △ Di-6S, 4 ; △ 4.5-unsaturated hexasacchride, 5 ; △4.5-unsaturated tetrasaccharide, D ; HA without enzyme treatment, O ; origin.

THERMOSTABILITY

The purified enzyme was heated at various temperature for 10 min and the residual activity against HA was determined. The enzyme was stable at 40°C, significant loss of activity was seen at 50°C, and almost complete loss occurred at 60°C (Fig. 5).

OPTIMAL pH

Enzyme activity was measured at various pH to estimate the optimal pH for reaction. All substrates were used at final concentration of 1 mg/ml. Two concentrations of the enzyme were used; 8×10^{-3} U against HA and Ch, and 30×10^{-3} U against ChS-A, and ChS-C. Buffers employed were acetate buffer (pH 4.0 to 5.5), phosphate buffer (pH 6.0 to 7.0), and Tris-HCl buffer (pH 7.5 to 8.5). Final concentration of the buffers was 0.1 M. The activity was measured by increase of absorbance at 232 nm. Fig. 6 shows that optimal pH was found at pH 5.5 in all the substrates.

EFFECTS OF METAL IONS

Effects of some metal ions on the enzymatic activity against HA was assessed at a concentration of 10^{-3} M. Activation was found in Ca^{2+}, Mg^{2+}, and Mn^{2+}. Cu^{2+} and Hg^{2+} inhibited completely. (Table 2).

PAPER CHROMATOGRAPHY OF THE REACTION PRODUCTS

The liberated sugars from each substrate by the purified enzyme were compared using paper chromatography. From HA, a sugar, whose Rf value (0.27) was the same as that of authentic ΔDi-OS (2-acetamido-2-deoxy-3-*o*-(β-D-gluco-4-enepyranosyluronic acid)-D-galactose), was detected (Fig. 7). However, this product sugar is thought to be unsaturated disaccharide lacking sulfate group as ΔDi-OS, probably it may be ΔDi-GNAc(β) (2-acetamido-2-deoxy-3-*o*-(β-D-gluco-4-enepyranosyluronic acid)-D-glucose). From ChS-A and ChS-C, two kinds of sugars were detected (Fig. 7). One kind represented the Rf value of 0.12. This value was the same as ΔDi-6S (2-acetamido-2-deoxy-3-*o*-(β-D-gluco-4-enepyranosyluronic acid)-6-*o*-sulfo-D-galactose). Another sugar showed Rf value of 0.18 which was corresponded to that of Di-4S (2-acetamido-2-deoxy-3-*o*-(β-D-

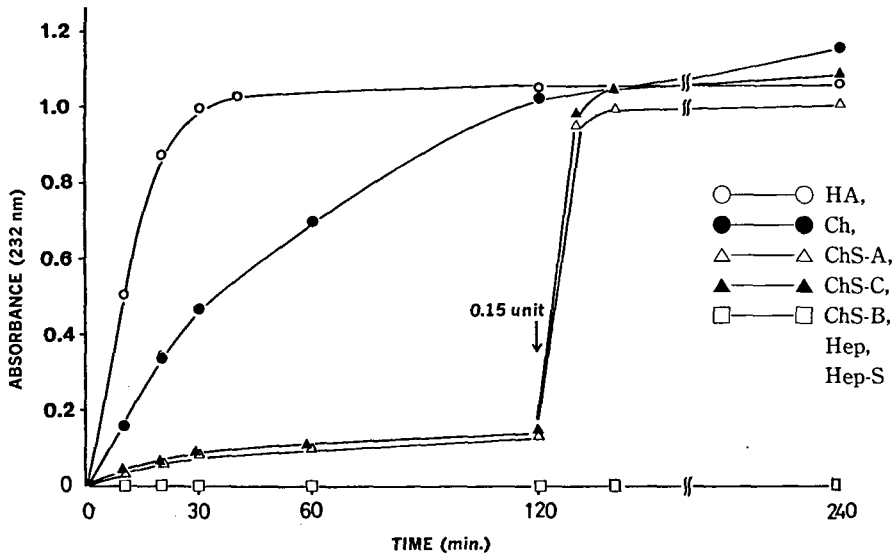

Fig. 9 : Comparison of rates of degradation of various mucopolysaccharides by hyaluronidase

gluco-4-enepyranosyluronic acid)-4-*o*-sulfo-D-galactose). From the degradative product of Ch, a sugar showed a Rf value of 0.27. This value was quite common to ΔDi-OS. No free sugar was detected from the reaction products of ChS-B, Hep, and Hep-S.

When HA was digested with commercially available hyaluronidase and the degradation products were developed by the same way, Δ4.5-unsaturated tetrasaccharide (Rf=0.034) and Δ4.5-unsaturated hexasaccharide (Rf=0.11) were found (Fig. 8), which, however, were not seen from the digestive products by *P. acnes* hyaluronidase.

DEGRADATION BY *P. ACNES* HYALURONIDASE OF SOME ACID MUCOPOLYSACCHARIDES

Kinetics of degradation of acid mucopolysaccharides by hyaluronidase was examined photometrically at 232 nm. Concentration of each substrate was adjusted to 2 μmole/ml equivalent to uronic acid and hexosamine. Enzyme concentration was 8×10^{-3} U. As illustrated in Fig. 9, degradation of HA was rapid till 30 min. Degradation of Ch continued longer, but with lower rate than that of HA. Only a little increase of UV absorbance was observed in ChS-A and ChS-C. However, when the concentration of enzyme in the two reaction mixtures was increased to 150×10^{-3} U at 120 min after start of incubation, absorbance increased dramatically. In ChS-B, Hep, and Hep-S, the similar effect of the concentration of the enzyme was not observed.

When the degradation rate by hyaluronidase of HA was supposed to be 100%, the relative rates against Ch, ChS-A, and ChS-C by this enzyme were calculated to be 47%, 8%, and 8%, respectively. These results indicate that hyaluronidase of *P. acnes* was most active against HA and moderately against Ch. ChS-A, and ChS-C were rather resistant to this enzyme.

DISCUSSION

It was preliminarily reported that many strains of *P. acnes* isolated from lesions of periodontal diseases degraded HA an Ch[14),15)]. In these reports, assay of enzyme activity was carried out by titration method with toluidine blue solution or by acid precipitation of the residual undigested substrates in agar plates containing substrates and the enzyme preparation, developed by Hoeffler[7)], and Smith and Willet[19)]. Detailed enzymatic properties were not studied by these authors.

We demonstrated in the present paper that hyaluronidase liberated Δ4.5-unsaturated disaccharides from HA, indicating that hyaluronidase from the oral strain of *P. acnes* has common enzymatic nature to other bacterial origin hyaluronidase[10)].

Our enzyme did not show the strict substrate specificity, it degraded also Ch, ChS-A, and ChS-C other than HA. These properties of substrate spectrum were found in other hyaluronidase preparation[3),7),8)]. Ingham et al[8)]. reported that molecular weight of *P. acnes* hyaluronidase was 85,000. This value is somewhat larger than our result (67,000).

In our previous paper[22)], we described that an oral strain of *Bacteroides* sp. produced enzyme(s), responsible for breakdown of the various mucopolysaccharides, including Hep and Hep-S. However, the enzyme preparation presented here did not react to these two substrates.

Conceivable roles of hyaluronidase produced in infectious lesions are primarily to be spreading factor of the enzyme[1)]. Relationship between development of periodontal diseases by the oral strains *P. acnes* and the roles of enzyme degrading the constituents of the connective tissue remains to be investigated in future.

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