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PZ-peptidase activities in *Streptococcus sanguis* and other oral bacteria

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*Streptococcus sanguis*와 여타 구강세균이 생산하는 PZ-peptidase 활성

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ABSTRACT

The occurrence of PZ-peptidase in *Streptococcus sanguis* and other oral bacteria was investigated utilizing washed whole cells as the enzyme source and PZ-pentapeptide as its substrate. Under the culture conditions employed in the present study, *Streptococcus sanguis* strains, fresh isolates as well as laboratory strains, produced a broad range of the enzyme activity (0.5~7.9 unit/mg protein). The strains of both *Streptococcus mutans* and *Lactobacilli* showed low levels of activity (0~0.5 unit /mg protein for *S. mutans*). As compared with the enzyme activities of other bacteria, a moderate range of activity was produced by the strains of *Streptococcus mitis* and *Streptoccus salivarius*. *Actinomyces* strains, like those of *S. sanguis*, produced a varying amount of activity (0~9.8 unit/ mg protein). A possible involvement of the oral bacterial PZ-peptidase in the metabolism of human saliva proteins is discussed.

INTRODUCTION

PZ-peptidase (EC 3, 4, 24, 3) is an enzyme capable of cleaving endohydrolytically 4-phenylazobenzyloxycarbonyl-L-Pro-L-Leu-Gly-L-Pro-D-Arg (designated as PZ-peptide) at the bond between L-Leu and Gly.

This compound was synthesized originally for monitoring collagenolytic activity of *Clostridium histolyticum* (Wunsch and Heidrich, 1963), and has been widely used in searching for bacterial and animal proteases with collagenase-like specificity (Lecroisey *et al.*, 1975 ; Strauch *et al.*, 1968).

Recently it has been shown, however, that animal collagenase and PZ-peptidase were separate enzymes and that the peptidase had no action on native collagen molecules (Harper and Gross, 1970 ; Harris and Krane, 1972). No bacterial PZ-peptidase which is detectible independently of collagenase activity has been reported. In the present paper, the author reports the PZ-peptidase activities of major oral bacteria which are not associated with collagenase, and a possible involvement of this enzyme in the metabolism of human saliva proteins are discussed.

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MATERIALS AND METHODS

Bacterial strains and growth conditions

The laboratory strains and fresh isolates of *S. sanguis* utilized in the present study were described previously (Choih *et al.*, 1979). *S. mutnas* strains 6715, ING, BHT, SL-1 and LM-7 were obtained from Dr. A. L. Coykendall. *Actinomyces viscosus* ATCC 15987, P1, P2, and *Actinomyces naeslundii* ATCC 12104, N9, C2 were obtained form Dr. R. P. Ellen.

Isolates of *S. mutans*, *S. salivarius*, *Actinomyces* and *Lactobacilli* were obtained from whole human saliva. *Streptococcus* strains were isolated on Mitis-Salivarius agar based on the criteria of Carlsson(1972). Strains of *Actinomyces* and *Lactobacilli* were isolated on CNAC-20 (Ellen and Bacerzak-Raczkowski, 1975) and Rogosa SL agar medium (Difco Lab., Detroit, U.S.A.), respectively.

Actinomyces medium was consisted of (per liter): trypticase soy broth, 27g; yeast extracts, 5g; and glucose, 5g. The autoclaved medium was supplemented with 10m*l* of sterile 1M Na_2CO_3, and cultures were grown aerobicalle at 37°C for 48h.

Source of enzyme

Bacterial cells were harvested by centrifugation (10,000×g, 20min, 4°C) and washed three times with 0.15M saline solution. Washed cells were suspended in the same saline solution and used for the enzyme assay.

Assays of PZ-peptidase and collagenase activities

The method of Wunsch and Heidrich (1963) was modified as follows. PZ-peptide (Sigma Chemical Co.) was dissolved in 2 mg/m*l* in 0.05M Tris-HCl buffer, pH 7.4. The reaction mixture contained 0.1 m*l* of Tris-HCl buffer (0.05M), pH 7.4 and 0.05m*l* of bacterial cells of approximately70μg of protein. Incubation was carried out for 90 min at 37°C with constant shaking. The reaction was stopped by adding 0.2lm*l* of 5% citric acid. Blanks were prepared by incubating bacterial cells and substrate separately.

The bacterial cells in the reaction mixture were removed by centrifugation, and the supernatants were extracted with 1m*l* of ethyl acetate by vortexing for 1 min and centrifuged briefly to remove water droplets from the organic phase. The extracts were read at 320 nm for product estimation.

The identification of the cleavage products from the substrate was shown by thin-layer chromatography on silica gel G (Analtech. Inc., Newark, U.S.A.), with n-butanol-acetic acid-water (4 : 1 : 1, by vol.) system using authentic PZ-Pro-Leu (Sigma Chemical Co., St. Louis, U.S.A.).

A unit of activity is defined as a change in absorbance of 1.0 up on incubation at 37°C for 90 min.

Collagenase activity was assayed against insoluble bovine Achilles tendon collagen according to the procedure of Mandl *et al.*(1953). Cell proteins were determined according to Lowry *et al.* (1951) after 1m*l* of cells was hydrolyzed with 10 m*l* of 1N NaOH for 60 min at room temperature.

RESULTS

The enzyme activity from *S. sanguis* strain 903 cells was proportional to the cell concentration (Fig. 1), and its release from the cells as measured by its activity was linear with respect to the time of incubation (Fig.2). In subsequent experiments, enzyme activity determinations were all conducted within the linear portion of the curve.

PZ-peptidase activity from *S. sanguis* strains.

Table I shows the PZ-peptidase activities from various strains of *S. sanguis*. Both labo-

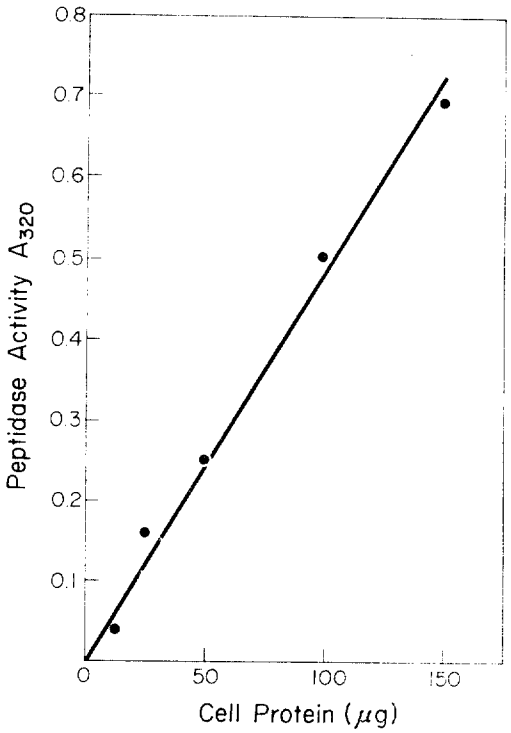

Fig. 1. Enzyme activity vs. the cell protein representing the bacterial cell numbers utilized in the assays. PZ-peptidase activity of *S. sanguis* strain 903 cells was measured as described in the text.

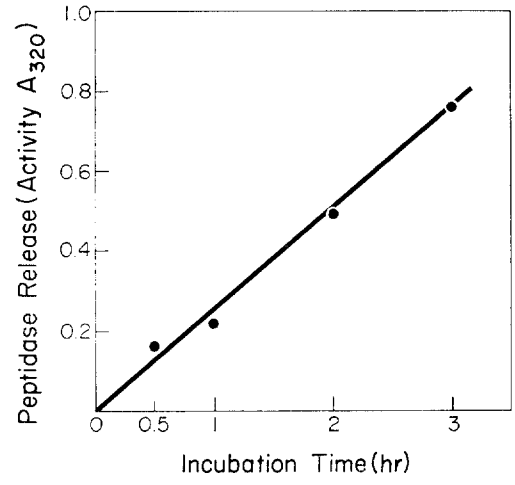

Fig. 2. Time-dependent release of PZ-peptidase during incubation in the reaction mixture as measured by its activity. *S. sanguis* strain 903 cells were used as the enzyme source as described in the text.

Table 1. PZ-peptidase activities in *S. sanguis* strains

Strains	PZ-peptidase activity (Unit*/mg protein)
903	5.0
10557	0.5
9811	6.5
10558	2.8
66×49	7.9
M5	6.4
J4	2.6
CB	1.1
#1	0.5

*A unit of activity=a change in absorbance of 1.0 upon incubation at 37°C for 90 min.

Table 2. PZ-peptidase activities in various oral bacteria

Oral bacteria tested		PZ-peptidase activity (Unit*/mg protein)
Streptococcus mutans	6715	N.D.**
	ING	0.5
	BHT	N.D.
	SL-1	0.2
	LM-7	0.4
	#307	0.1
	#309	0.3
Streptococcus mitis	1C-6	3.3
	31	1.7
	26	1.1
Streptococcus salivarious	G	6.4
	#1	1.8
Lactobacillus	#60	0.1
	#61	0.7
	#65	0.4
Fusobacterium nucleatum	ATCC 25586	1.1
Actinomyces	ATCC 151987	2.7
	P1	N.D.
	P2	N.D.
	N9	0.1
	ATCC 12104	N.D.
	C2	0.1
	#10	9.8
	B	3.6

* A unit of activity=a change in absorbance of 1.0 upon incubation at 37°C for 90 min.

**N.D.-Not detectable

A broad range of activities, from 0.5 to 7.9 ratory strains and fresh isolates were tested. unit/mg protein, was observed. Strain 64×49 produced the highest level of activity and, strains 10557 and #1 the lowest level. All other strains produced enzyme activities falling in these two extreme values.

PZ-peptidase activities in other major oral bacteria

The enzyme activities of several other oral bacteria were given in Table II. *S. mutans* strains showed low activities ranging 0~0.5 unit. Similarly, low enzyme activities were produced by lactobacilli. Strains of *S. mitis* and *S. salivarius* produced high levels of activity, but the activities were generally lower than those of *S. sanguis*.

Oral *Actinomyces* produced also a wide range of activities. Strains such as #10, B and ATCC 15987 produced high levels and other strains showed very low activities or none.

Since the substrate PZ-peptide has been used for the purpose of detecting collagenase-like enzymes, the collagenase production from these bacteria was tested. No activity was detected.

DISCUSSION

The data demonstrate the existence of an enzyme activity in human oral bacteria that is capable of cleaving PZ-peptide. The separation and identification of the reaction products by thin layer chomatography provided additional support for the enzymatic nature of the reaction (data not shown). Since the intact bacterial cells were used as enzyme sources, the linearity of the reaction curve implied that the cell-associated enzyme, being either cellwall bound or a cytosol component, was released continuously during the incubation (Fig. 2.)

The C-terminal D-arginine of the PZ-peptide makes it resistant to nonspecific proteases such as trypsin, chymotrypsin, carboxypeptidase A and B (Wunsch and Heidrich, 1963).

Collagenolytic activity was not detected from the bacteria tested as they are known to be non-collagenolytic, thus eliminating its involvement in the breakdown of the peptide.

Large variations in PZ-peptidase activity were observed among groups and among strains within a group. *S. mutans* and *Lactobacilli* are low in the activity. Among *Actinomyces*, fresh isolates (#10 and B) produced higher activity as compared to other loboratory strains with an exception of ATCC 15987. Whether or not fresh isolates are generally high in activity was not studied further.

Strains of *S. sanguis* produced much higher and, among strains, less variable levels of enzyme as compared to other groups. This coincides with the observation that *S. sanguis* produced high level of arylaminopeptidase, whereas extremely low activity was obtained from *S. mutans* (Oya *et al.*, 1971). The arylaminopeptidase has been detected in human whole saliva and its activity was attributed to oral bacteria (Makinen, 1966; Oya. *etal.*, 1968). In the present study, *S. sanguis* strain 10577 among laboratory strains studied, produced the lowest level of enzyme. This strain is known to behave abnormally among members of *S. sanguis*, and it was suggested that this strain should not be assigned to sanguis species (Cole and Kolstad, 1974).

It has been reported that human parotid saliva contains a complexity of proline-rich proteins (Hay and Oppenheim, 1974 ; Bennick, 1977) and these proteins are, once secreted into oral cavity, subject to marked degradation (Hay and Gron, 1976). Choih *et al.* (1979) have demonstrated that *S. sanguis* may be at least partially responsible for the in vivo modification of the proline-rich proteins from the parotid gland. Bennick (1977) has reported, from amino acid sequence data, that acidic

proline-rich proteins A and C as well as basic proteins contained the sequence-Pro-x-Gly-Pro-wh-ich is analogous to the sequence of PZ-peptide and some sequences in collagen molecules.

Although sthe enzyme(s) involved in the degradation of proline-rich proteins from parotid gland is not entirely known, the specificity of PZ-peptidase and the known sequence of the amino acids in proline-rich proteins (Bennick, 1977) make it likely that PZ-peptidase is involved in such a degradation.

Cowman *et al.* (1978) have demonstrated that major oral streptococci *S. mutans*, *S. sanguis*, *S. salivarius* and *S. mitis* require certain amino acids for their growth. The free amino acid contents present in saliva are inadequate to support their growth (Hyatt and Hayes, 1975). Also extremely low levels of free amino acids are present in plaque(Critchley, 1969). There fore, the ability of streptococci to accumulate amino acids from other sources in their oral environment may be important to their survival. Alternate sources of amino acids such as the salivary and dietary proteins, and peptides would require further degradation by enzymes associated directly with the organisms or by other exogenous enzymes. Proteolytic activity has been observed in *S. sanguis*, *S. mutans* (Cowman *et al.*, 1976), as well as in *S. mitis* and *Actinomyces* (Choih, unpublished data). Also, *S. sanguis* and *S. mutans* have been shown to have the capability to utilize certain saliva proteins as nitrogen sources (Cowman *et al.*, 1976).

Therefore, the PZ-peptidase, together with proteases and arylamino-peptidases detected in oral bacteria, may be involved in obtaining amino acids as required nutrient by degrading salivary proteins and peptides. Whether the production of this enzyme is restricted to the oral bacteria or is occurring in other human indigenous bacteria awaits further studies.

摘 要

*Streptococcus sanguis*와 여타 구강세균의 PZ-peptidase의 생산을 연구하였다. 세척한 온전한 세균세포를 효소원으로, 그리고 PZ-pentapeptide를 효소의 기질로 사용하였다. 이 연구에서 책택한 균의 배양조건에서, *S. sanguis*에서는 넓은 범위의 효소활성도가, 실험실 균주와 신선한 분리균주에서 검출되었는데, 그 값은 0.5~7.9Unit /mg protein 이었다.

*Streptococcus mutans*와 *Lactobacilli*는 낮은 효소활성을 보였고 *S. mutans*의 경우 그 값은 0~0.5Unit/mg protein이었다.

*Streptococcus mitis*와 *Streptococcus salivarius*는 다른 세균과 비교할 때 중등도의 효소활성을 갖고 있었고, *Actinomyces*의 균주들은 *S. sanguis*처럼 넓은 범위의 활성도(0~9.8 unit/mg protein)를 지니고 있었다.

본 논문에서 취급한 구강세균이 생성하는 PZ-peptidase가 사람의 타액단백질의 분해에 참여할 수 있는 가능성을 더불어 고찰하였다.

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