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Hydrolysis of γ-Glutamyl Linkages by *Fusobacterium nucleatum*

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Abstract. The cell extracts of two human oral strains (FN2 and FN3) of *Fusobacterium nucleatum* displayed exceptionally high γ-glutamylpeptidase activity as determined with *N*-γ-L-glutamyl-2-naphthylamine as substrate. This activity was so dominant that the hydrolysis of other *N*-aminoacyl-2-naphthylamines progressed at a rate <10% of the former. Two major enzymes (I and II) were partially purified from FN2. I had a molecular weight of 115,000 and did not hydrolyze γ-glutamylcysteinylglycine (glutathione). II had a molecular weight of 70,000 and rapidly liberated only glutamic acid from glutathione. Strain FN3 contained several enzymes hydrolyzing γ-glu-2NA. Direct anion exchange chromatography of FN3 cell extracts separated one enzyme that liberated both glutamic acid and glycine from glutathione, one that was inactive against glutathione (but hydrolyzed γ-glu-2NA), and one that liberated only glutamic acid. Although γ-glu-2NA was a good synthetic substrate, glutathione was hydrolyzed at least 500 times faster by an enzyme present in both strains. These results indicate that the presence of γ-glutamylpeptidase activity is very characteristic of these *F. nucleatum* strains.

The fusobacteria are Gram-negative, non-spore-forming rods that have been found in various cavities of man and other animals [5]. In addition to metabolizing carbohydrates, these organisms may also metabolize peptone. *Fusobacterium nucleatum* has been frequently isolated from or demonstrated in the human oral cavity [4, 8, 17, 20, 25], and proteolytic activity present in clinical isolates of *F. nucleatum* has been reported [6, 14, 21, 27]. However, the enzymology of human oral fusobacteria has not been thoroughly investigated. Human oral fusobacteria may be important organisms also in the metabolism of cysteine, methionine, and other sulfur-containing compounds present in the oral cavity [22], thus possibly contributing to the production of volatile, malodorous substances. Of special interest is a study [24] showing that resting cells of *F. nucleatum* ATCC 10953 failed to accumulate glucose, and that the addition of glutamic acid, lysine, or histidine to anaerobic suspensions of cells caused a rapid accumulation of glucose. In other studies a major cell envelope protein from *F. nucleatum* was isolated and suggested to participate in cell surface-related interactions of this organism [2].

Our preliminary studies showed that the cells of *F. nucleatum* (human oral strains FN2 and FN3) produce peptidases that hydrolyze γ-glutamyl-2-naphthylamine (γ-glu-2NA) at a high rate and that are especially active on glutathione (γ-Glu-Cys-Gly), hydrolyzing the γ-Glu-Cys or the Cys-Gly bonds in the latter. The high activity of these organisms on γ-glutamyl "peptide" (or amide) bonds also raises the question whether the cells use this mechanism to increase the availability of glutamic acid, which is reportedly needed in glucose intake [24], or whether the hydrolysis of these bonds is primarily related to thiol metabolism. This paper summarizes results from two studies that exemplify the ability of *F. nucleatum* to hydrolyze γ-glutamyl linkages. In the first study, two enzymes (γ-glutamylpeptidases; γ-GP) hydrolyzing γ-glu-2NA were partially purified from *F. nucleatum* strain FN2. One of the enzymes hydrolyzed glutathione. In the second study on strain FN3, several enzymes hydrolyzing γ-Glu-2NA were detected, two of the enzymes being active on glutathione.

Materials and Methods

Source and cultivation of microorganisms. The cells of *Fusobacterium nucleatum* (strains FN2 and FN3, isolated from the subgingival plaque of periodontitis patients) were maintained with biweekly transfers in Schaedler broth. Screw-capped flasks containing 500-ml aliquots of Schaedler broth were inoculated with *F. nucleatum* cultures and incubated anaerobically at 35°C for 72

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Table 1. Specific activity of the cell extracts (after sonication and centrifugation) of two *Fusobacterium nucleatum* strains and activity of the supernatant fluid of the culture to hydrolyze peptidase substrates

	FN2		FN3	
Substrate	Cell Extract	Cell Medium	Extract	Medium
$N\gamma$-L-Glutamyl-2NA[a]	0.91	—[b]	1.10	—
N-α-L-Arginyl-2NA[a]	0.04	—	0.11	—
BANA[a]	0.01	—	0.06	—
$N\alpha$-L-Prolyl-2NA[a]	<0.01	—	—	Traces
Azocoll[c]	6.7	—	9.1	+
Azocasein	—	—	Traces	—
Elastin–orcein	—	+	—	—
PZ-PLGPA[a]	0.4	—	0.4	—

[a] In μmol mg^{-1} min^{-1} $\times 10^3$.
[b] + denotes presence of activity in the filtrate and – indicates that no measurable hydrolysis was observed in 17 h.
[c] In enzyme units mg^{-1} min^{-1} (units defined in Calbiochem-Boehring Doc. No. 3805-880).

h. The cells were harvested by centrifugation for 10 min at 13,500 *g*, washed three times with cold 0.1 *M* Tris-HCl (pH 7.2, 1 m*M* $CaCl_2$), and finally resuspended in the same buffer. The suspensions were kept in an iced water bath and treated for 3 min (with intermittent cooling after every 30 s), with a Sonifier Cell Disrupter (Model W 1850D, Branson Sonic Power Company, Plainview, New York). The sonicates were centrifuged as above, and the supernatant fluids were studied for proteolytic activity. The purity of the cells was checked by phase contrast microscopy and by culturing on an enriched trypticase soy agar.

Chromatography. Gel filtration on Sephadex G-200 gel was carried out at 4°C under conditions specified below. Fast protein liquid chromatography (FPLC) was performed at 22°C with a Pharmacia (Uppsala, Sweden) unit and a Mono Q HR5/5 anion exchange column and Superose 6 and 12 gel columns. All samples injected into the FPLC unit were treated with 0.2-μ Acro LC13 filters (Gelman, Ann Arbor, Michigan). Amino acid analyses of the hydrolysis products of glutathione were performed on a Beckman Model 6300 Automated Amino Acid Analyzer.

Chemical methods. The enzyme activities were determined with *N*-aminoacyl-2-naphthylamines (2NAs), Azocoll, Azocasein, Elastin-Orcein, *N*-α-benzoyl-DL-arginyl-2NA (BANA) and phenylazobenzyloxycarbonyl-L-prolyl-L-leucylglycyl-L-prolyl-D-arginine (PZ-PLGPA) as substrates [13]. The protein concentrations were assayed with the Bio-Rad assay system [1] and in chromatography at 280 nm or 214 nm. Unless otherwise indicated, all substrates were obtained from Sigma. The water used in the study was prepared with a Millipore Milli-Q system and had a resistivity of 18 MΩ cm^{-1}.

The hydrolysis of glutathione was studied in a reaction mixture consisting of 1.0 ml of a 0.1 m*M* glutathione solution (in 50 m*M* phosphate buffer, pH 7.0) and of 10–50 μL of enzyme. Appropriate blanks, omitting either the enzyme or substrate, were included. The mixtures were incubated for various periods of time at 37°C. After incubation, the mixtures were immediately chilled and passed through an Amicon Centricon-10 filter for the removal of proteins. The protein-free filtrates were analyzed for the amino acids present in glutathione.

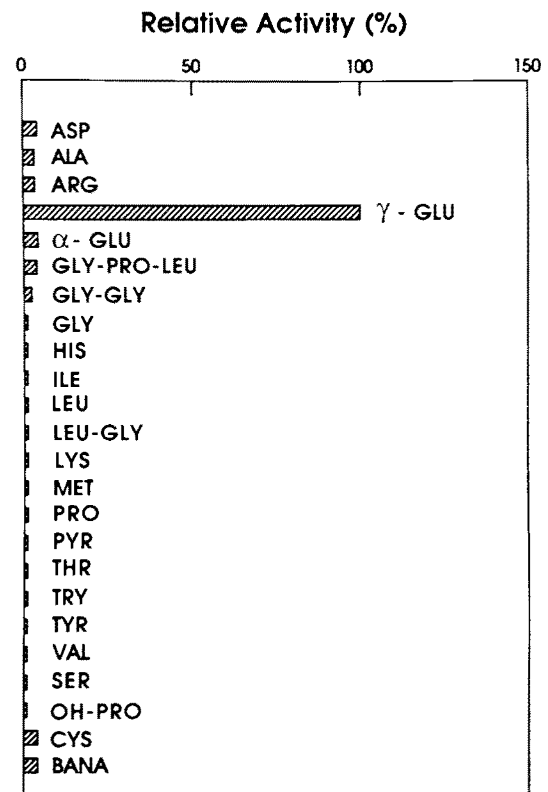

Fig. 1. Relative rate of the hydrolysis of various *N*-aminoacyl-2NAs by the centrifuged (10 min, 13,500 *g*) cell sonicate of *Fusobacterium nucleatum* (strain FN2). Tested in 50 m*M* Tris-HCl, pH 7.0, at 0.167 m*M* substrate concentrations (30°C). The rate of the hydrolysis of γ-glu-2NA is marked as 100%.

The partially purified γ-GPs of *F. nucleatum* FN3, which hydrolyzed glutathione, were subjected to preliminary chemical modification studies for the elucidation of the nature of the enzymes. The modifiers (Sigma) were tested in selected conditions (Table 4) previously used in the modification of specific amino acid residues present in enzymes [9–12]. The following reagents were tested: tetranitromethane (TNM; for nitration of active site of enzymes) [26], trinitrobenzenesulfonic acid (TNBSA; a relatively specific reagent of active lysyl residues) [15], *N*-ethoxycarbonyl-2-ethoxy-1,2-dihyroquinoline (EEDQ; highly specific for active carboxyl groups) [23], 2,3-butanedione (BD; used to modify active site arginyl groups under non-photochemical conditions) [11], diethylpyrocarbonate (DEP; for carbethoxylation of certain active site groups) [16], and 5,5′-dithiobis-2-nitrobenzoic acid (DTNBA; a specific reagent for enzyme SH-groups [3].

Results and Interpretations

Proteolytic activity of crude extracts. The extracts of both strains of *Fusobacterium nucleatum* exhibited essentially similar peptidolytic patterns (Table 1). γ-

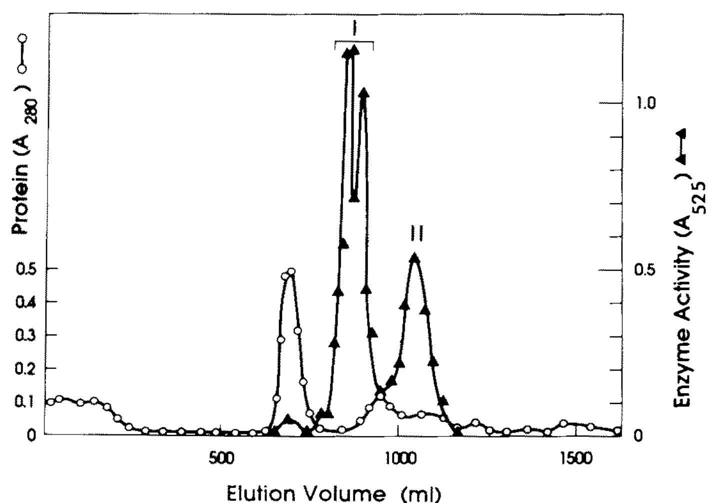

Fig. 2. Separation of two major γ-GPs of *F. nucleatum* (strain FN2) on a Sephadex G-200 column (5 cm × 88 cm). The column was eluted with 50 m*M* Tris-HCl, pH 7.8 (containing 1 m*M* $CaCl_2$) at a flow rate of 0.5 ml/min. The active fractions (tested with γ-glu-2NA) were pooled as follows: enzyme I (double peak) 800–970 ml; enzyme II, 975–1,150 ml. The sample applied to the column was a 25-ml centrifuged cell extract containing 82.5 mg protein.

Glu-2NA was hydrolyzed at a high rate. The insoluble proteinase substrate Azocoll was hydrolyzed at a clearly measurable rate, and PZ-PLGPA, a soluble substrate frequently used in the study of bacterial collagenolytic enzymes [10], was also hydrolyzed. Most of the proteolytic activity was associated with cells. Figure 1 shows the relative ability of the sonicate from *F. nucleatum* FN2 to hydrolyze *N*-aminoacyl-2NAs. The dominance of the hydrolysis of γ-glu-2NA was striking. A close homologue, *N*-α-L-glu-2NA, was hydrolyzed at a much lower rate.

Partial purification of γ-GPs of FN2. The cell extracts were subjected to gel filtration on a Sephadex G-200 column. Two major enzymes (I and II) with γ-GP activity were revealed (Fig. 2), the fraction with the higher molecular weight exhibiting two maxima. Because of the incomplete separation of these high-molecular-weight enzymes, the active fractions (800–970 ml) were combined for the next purification step. The fractions comprising both major enzymes (I and II; Fig. 2) were thus pooled; the pools (175 ml each) were concentrated 20-fold and dialyzed for 48 h at 4°C against 1000 volumes of 10 m*M* Tris-HCl, pH 7.8 (containing 1 m*M* $CaCl_2$). The two dialysates were subjected to FPLC on a Mono Q anion exchange column with the NaCl gradient indicated in Fig. 3. The major enzyme peak shown in Fig. 3A was designated as γ-GP I, although it was not established from which of the two close peaks shown in Fig. 2 it was derived. The low-molecular-weight enzyme (Fig. 2), designated as γ-GP II, was separated as shown in Fig. 3B. The active fractions of the two major γ-GPs (I and II) were pooled, dialyzed as above for the removal of excess NaCl, and subjected to glutathione studies. The molecular weights of enzymes I and II, estimated by chromatography on Sephadex G-200 gel, were 115,000 and 70,000, respectively. Table 2 shows the specific activity of the enzymes at various stages of purification.

Hydrolysis of glutathione by FN2 enzymes. Only enzyme II hydrolyzed glutathione, the scissle bond being γ-Glu-Cys (Fig. 4). Amino acid analysis showed that free glutamic acid and cysteinylglycine accumulated in the reaction mixture at a steady rate and at a stoichiometric ratio for up to 17 h. No free glycine or cysteine were detected. Direct comparisons between substrates showed that although γ-glu-2NA was a good substrate, glutathione was hydrolyzed by enzyme II at a rate much faster than γ-glu-2NA. This is shown below with strain FN3.

Separation of glutathione-hydrolyzing enzymes from FN3. The second study carried out on strain FN3 showed that a single FPLC on a Mono Q column separated three γ-GPs adequately (Fig. 5). Two of the enzymes (11 and 16 min) had sufficient activity for characterization studies (see below). This anion exchange FPLC thus separated one enzyme (11 min) that simultaneously hydrolyzed both the Glu-Cys bond (liberating glutamic acid) and the Cys-Gly bond (liberating glycine) of glutathione, and another enzyme (16 min) that hydrolyzed the Glu-Cys bond only (liberating glutamic acid). Neither enzyme liberated free cysteine from glutathione. The enzyme eluted at 14 min (middle peak in Fig. 5) did not hydrolyze glutathione.

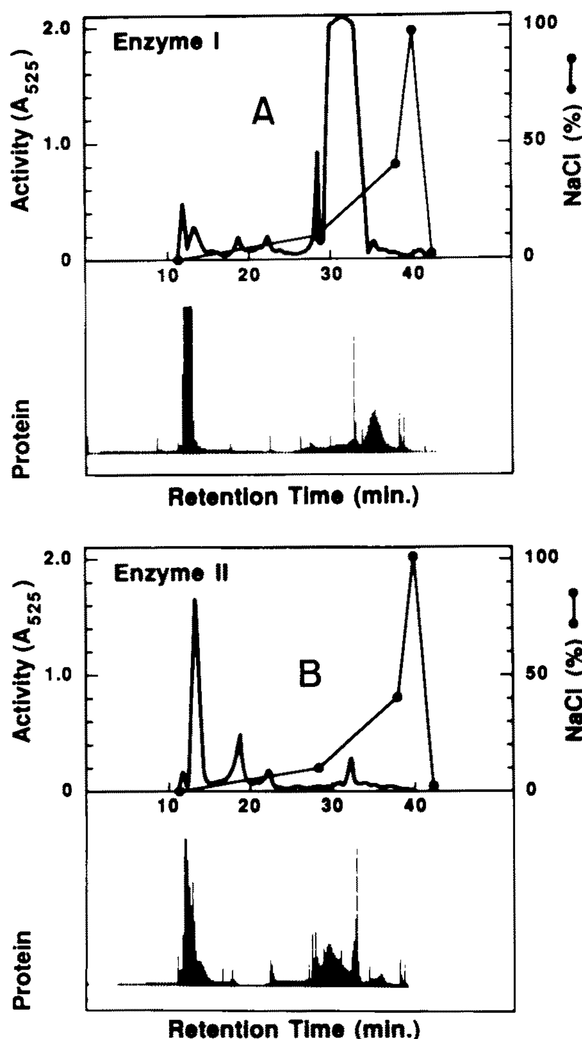

Fig. 3. Separation of γ-GPs of *F. nucleatum* (strain FN2) with FPLC. The enzymes (I and II) from the previous step (Fig. 2) were dialyzed (see text) and injected into a Mono Q anion exchange column. A NaCl gradient was applied as shown (100% NaCl = 1.0 *M*). The elution buffer was 50 m*M* Tris-HCl, pH 7.8 (containing 0.1 m*M* $CaCl_2$) and the flow rate was 2.0 ml/min. The active fractions (tested with γ-glu-2NA) were pooled as follows: enzyme I (upper panel), 29–33 min.: enzyme II (lower panel), 12–14 min. Protein was monitored at 280 mm. In the protein printout the maximum peak height corresponds to 1.0 AUFS.

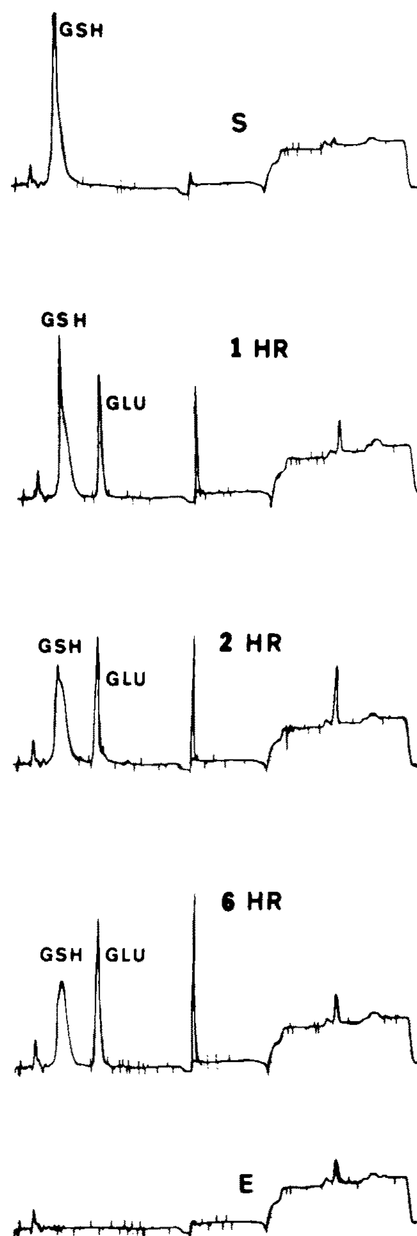

Fig. 4. Hydrolysis of glutathione (GSH) by *F. nucleatum* (strain FN2) enzyme II, 0.1 m*M* glutathione was incubated for up to 6 h with the enzyme at 37°C. Aliquots of the reaction mixtures and the blanks (omitting either enzyme or substrate) were subjected to amino acid analysis. Glutathione was hydrolyzed into glutamic acid and cysteinylglycine, while the substrate blanks (S) yielded the glutathione peak only, the enzyme blanks (E) showing no significant reaction with ninhydrin.

Properties of two glutathione-hydrolyzing enzymes of FN3. The two major (11-min and 16-min) γ-GPs that were revealed by their hydrolysis of γ-glu-2NA (Fig. 5) hydrolyzed glutathione very rapidly (Table 3), the enzyme with the ability to liberate both glutamic acid and glycine being especially active on glutathione. In no case was free cysteine detected in reaction mixtures. Direct comparison between substrates showed that glutathione was hydrolyzed by both enzymes much faster than γ-glu-2NA. α-Glu-Glu and α-Glu-Gly-Phe were also hydrolyzed. The following substrates were not hydrolyzed, or the rate of hydrolysis by both enzymes was low: Arg-Gly (at low rate), Arg-Gly-Gly (Arg-Gly bond

Table 2. Purification of two γ-GPs (I and II) from the cells of *Fusobacterium nucleatum* (strain FN2)[a]

Purification step	Volume (ml)	Protein (mg/ml)	Total protein (mg)	Specific activity (μmol mg^{-1} min^{-}) × 10^3
Cell sonicate (after centrifugation)	25	3.3	82.5	0.91
After Sephadex G-200 I	175	0.047	8.25	7.12
II	175	0.085	14.9	5.42
After concentration I	18	0.41	7.45	13.1
II	17	0.68	11.4	5.5
After Mono Q separation				
I (0.2 *M* NaCl)[b]	10	0.05	0.5	42.0
II (0.03 *M* NaCl)	6	0.175	1.0	7.6

[a] The enzymes were designated as I and II in the order of elution from the Sephadex G-200 column (Fig. 2).
[b] The figures show the approximate NaCl levels needed to deabsorp the enzyme.

hydrolyzed at low rate), Pro-Gly (no hydrolysis), Pro-Gly-Gly (low rate at the Pro-Gly bond), Pro-Gly-Gly-Gly (no hydrolysis), Leu-Gly-Gly, hippuryl-Phe and hippuryl-Arg (no hydrolysis). These results suggest that γ-Glu-Cys-Gly (glutathione) may be a specific in vivo substrate of these enzymes. The optimum pH for the liberation of glutamic acid by both enzymes was near pH 8, whereas the liberation of glycine occurred most rapidly around pH 7.4. $CaCl_2$ (1 m*M*) had no effect on these hydrolyses, and EDTA had no effect on the reactions when tested even at a relatively high 10-mm concentration. On the other hand, TNM almost totally inactivated the enzymes. TNBSA, EEDQ, and BD were also relatively potent inactivators, but DEP and DTNBA failed to cause any significant effect under these conditions (Table 4).

On the basis of earlier literature and experience [3, 9–13], it appears that the glutathione-hydrolyzing enzymes of strain FN3 do not need SH groups for full activity. The inactivations of the enzyme by TNM and EEDQ were sufficiently fast processes to indicate the presence of active tyrosyl and carboxyl groups in the enzymes. The reactions caused by TNBSA and DEP at the pH values shown (Table 4) indicate the possible involvement of lysyl but not histidyl residues. In other studies BD has reacted, under nonphotooxidative conditions, with arginyl residues [11]. These residues may not be necessary for the activity of the present enzymes.

The metabolic role of the present γ-GPs is not known, but the high activity of some of the enzymes on glutathione suggests that they may be important to the growth and metabolic control of these organisms. Glutathione is a submajor constituent of all cells and is almost always the major nonprotein thiol present in cells [7]. As a SH-carrier and coenzyme, glutathione plays a controlling role in oxidation-reduction, biosynthetic pathways, cell division, and other phenomena. It is also involved in transpeptidation as a glutamyl donor, in nitrate reduction, etc. However, glutathione must be regarded as a cellular component. It is not known whether the *Fusobacterium* enzymes described here are confined to the intracellular compartment of these organisms only, or whether they partly contribute to the breakdown of host tissue glu-

Table 3. Ability of *Fusobacterium nucleatum* FN3 γ-GPs to hydrolyze glutamic acid derivatives

Substrate	11-min enzyme (liberates Glu & Gly)[a]	16-min enzyme (liberates Glu only)[a]
γ-L-Glu-L-Cys-Gly	13,872 (for Glu)	672 (for Glu)
↑ or ↑	5,600 (for Gly)	
α-L-Glu-α-L-Glu	336	Traces
↓		
α-L-Glu-Gly-L-Phe	296	Traces
γ-L-Glu-2NA (Sigma)	26	13
α-L-Glu-α-L-Lys	Traces	—
γ-L-Glu-L-Leu	Traces	—
γ-L-Glu-α-L-Glu	—	—
γ-L-Glu-α-L-Gln	—	—
γ-L-Glu-Gly	—	—
γ-D-Glu-Gly	—	—
γ-L-Glu-ε-L-Lys	—	—
γ-L-Glu-L-His	—	—
α-L-Glu-2NA (Sigma)	—	—

[a] Connotes liberation of amino acids from glutathione.

The reactions were carried out in 25 mM Tris-HCl (pH 8.0) at 30°C with enzymes (11-min and 16-min) separated by FPLC (Fig. 5). The activities are given in nmol min^{-1} mg^{-1} (— indicates that no hydrolysis was observed). In tripeptides, the scissile bond is marked (arrow). Unless otherwise indicated, the substrates were obtained from Serva.

Table 4. Effect of chemical modification on the activity of the γ-GPs of *Fusobacterium nucleatum* FN3, which hydrolyze glutathione

Modifier (10 m*M*)	Conditions	% Activity remaining	
		Glu- and Gly-liberating enzyme (11 min)[a]	Glu-liberating enzyme (16 min)[a]
TNBSA	Tris-HCl, pH 9.0 (23°C)	38	21
TNM	Tris-HCl, pH 8.0 (23°C)	6	0
DEP	Phosphate, pH 6.0 (23°C)	100	92
EEDQ	Phosphate, pH 6.0	45 (40°C)	23 (23°C)[b]
BD	Tris-HCl, pH 8.0 (23°C)	45	25
DTNBA	Phosphate, pH 8.0 (30°C)	100	100

[a] Connote enzymes liberating amino acids from glutathione (Fig. 5).
[b] The treatment with EEDQ should preferably be carried out at 40°C. The Glu-liberating enzyme was unstable, however, at this temperature.

The enzymes were modified with the reagents indicated, the reactions were arrested and the modification mixtures dialyzed using Millipore "V" Series membranes (VSWP 025 00; 0.025 μm) for 2 to 3 h at +4°C to remove the excess of modifiers. The activity of the modified enzymes was tested using glutathione as substrate and analyzing the reaction products with an amino acid analyzer. The enzymes were obtained from FPLC on a Mono Q column (Fig. 5).

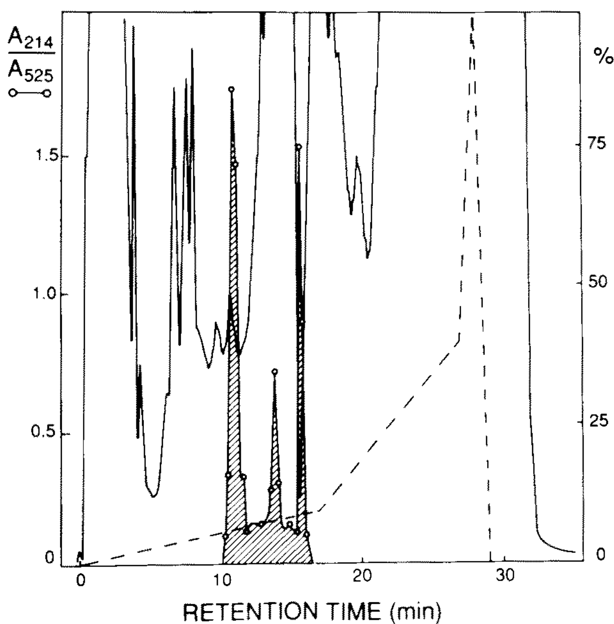

Fig. 5. FPLC on a Mono Q column of the γ-GPs of *F. nucleatum* (FN3); 4.0 ml of centrifuged and dialyzed cell extract was injected into the column, which was eluted with 10 m*M* Tris-HCl, pH 7.5. A NaCl gradient (---; from zero to 1.0 *M*; 1.0 *M* = 100%) was applied as shown. The fractions were tested with γ-glu-2NA (A_{525}). The shaded areas show the separation of three major γ-GPs. The 11-min fraction liberated either glutamic acid or glycine from glutathione (leaving a Cys-dipeptide intact), the 14-min fraction did not hydrolyze glutathione at all, and the 16-min enzyme liberated glutamic acid from glutathione.

tathione (or other γ-glutamyl peptides) present in the growth environment of the bacteria.

Conclusions

1. The cell extracts of *Fusobacterium nucleatum* (human oral strains FN2 and FN3) exhibited strikingly strong, selective enzyme activity on γ-glutamyl-2-naphthylamine and glutathione, indicating that these organisms contain highly active γ-glutamyl-peptidases.
2. Two to three enzymes were identified on the basis of their rapid hydrolysis of γ-glutamyl-2-naphthylamine and were partially purified from the above strains. Each strain contained one enzyme that was inactive on glutathione, while at least one enzyme was present that cleaved the Glu-Cys bond only. These enzymes did not liberate free cysteine from glutathione.
3. The molecular weights of the two major γ-glutamylpeptidases from *F. nucleatum* strain FN2 were 115,000 and 70,000.
4. The ready hydrolysis of glutathione and γ-glutamyl-2-naphthylamine by *F. nucleatum* suggests that the role of the respective enzymes is associated with the regulatory functions of glutathione itself.

Literature Cited

1. Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72:248–254
2. DiRienzo JM, Rosan B (1984) Isolation of a major cell envelope protein from *Fusobacterium nucleatum*. Infect Immun 44:386–393
3. Ellman GL (1957) Tissue sulfhydryl groups. Arch Biochem Biophys 82:70–77

4. Gibbons RJ, Socransky SS, Sawyer S, Kapsimalls B, MacDonald JB (1963) The microbiota of the gingival crevice of man. II. The predominant cultivable organisms. Arch Oral Biol 8:281–289
5. Holdeman LV, Kelley RW, Moore WEC (1984) Anaerobic gram-negative straight, curved and helical rods. Family I. *Bacteroidaceae Pribram* 1933, 10[AL]. In: Krieg NR, Holt IG, (eds) Bergey's manual of systemic bacteriology, vol. 1. Baltimore: The Williams and Wilkins Co. pp 602–662
6. Kato T, Ono M, Ikawa M, Okuda K, Takazoe I (1984) Proteolytic activities of gram-negative bacteria isolated from human periodontal lesions. Jpn Oral Biol 26:953–958
7. Kosower EM (1976) Chemical properties of glutathione. In: Arias IM, Jakoby WB (eds) Glutathione: metabolism and function. New York: Raven Press, p 103
8. Loesche WJ, Syed SA, Schmidt E, Morrison EC (1985) Bacterial profiles of subgingival plaques in periodontics. J Periodontol 56:447–456
9. Mäkinen KK, Mäkinen P-L (1982) Diketones as photosensitizing agents: applications to α-amino acids and enzymes. Photochem Photobiol 35:761–765
10. Mäkinen KK, Mäkinen P-L (1987) Purification and properties of an extracellular collagenolytic protease produced by the human oral bacterium *Bacillus cereus* (strain Soc 67). J Biol Chem 262:12488–12495
11. Mäkinen KK, Mäkinen P-L, Wilkes SH, Bayliss ME, Prescott JM (1982) Photochemical inactivation of *Aeromonas* aminopeptidase by 2,3-butanedione. J Biol Chem 257:1765–1772
12. Mäkinen KK, Mäkinen P-L, Wilkes SH, Bayliss ME, Prescott JM (1982) Chemical modification of *Aeromonas* aminopeptidase. Evidence for the involvement of tyrosyl and carboxyl groups in the activity of the enzyme. Eur J Biochem 128:257–265
13. Mäkinen KK, Syed SA, Mäkinen P-L, Loesche WJ (1987) Dominance of iminopeptidase activity in the human oral bacterium *Treponema denticola* ATCC 35405. Curr Microbiol 14:341–346
14. Mayrand D, Grenier D (1985) Detection of collagenase activity in oral bacteria. Can J Microbiol 31:134–138
15. Means GE, Congdon WI, Bender ML (1972) Reactions of 2,4,6-trinitrobenzene-sulfonate ion with amines and hydroxide ion. Biochemistry 11:3564–3571
16. Miles EW (1977) Modification of histidyl residues in proteins by diethylpyrocarbonate. Methods Enzymol 47:431–442
17. Moore WEC (1987) Microbiology of periodontal disease. J Periodont Res 22:335–341
18. Moore WEC, Holdeman LV, Smibert RM, Good IJ, Burmeister JA, Palcanis KG, Ranney RR (1982) Bacteriology of experimental gingivitis in young adult humans. Infect Immun 38:651–667
19. Moore WEC, Holdeman LV, Smibert RM, Hash DE, Burmeister JA, Ranney RR (1982) Bacteriology of severe periodontitis in young adult humans. Infect Immun 38:1137–1148
20. Moore WEC, Holdeman LV, Smibert RM, Cato EP, Burmeister JA, Palcanis KG, Ranney RR (1984) Bacteriology of experimental gingivitis in children. Infect Immun 46:1–6
21. Nitzan D, Sperry JF, Wilkins TD (1978) Fibrinolytic activity of oral anaerobic bacteria. Arch Oral Biol 23:465–470
22. Planotti R, Lacnette S, Dills S (1986) Desulfuration of cysteine and methionine by *Fusobacterium nucleatum*. J Dent Res 65:913–917
23. Pougeois R, Satre M, Vignais PV (1978) N-Ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline, a new reagent of the mitochondrial F_1-ATPase. Biochemistry 17:3018–3023
24. Robrish SA, Oliver C, Thompson J (1987) Amino acid-dependent transport of sugars by *Fusobacterium nucleatum* ATCC 10953. J Bacteriol 169:3891–3897
25. Slots J (1979) Subgingival microflora and periodontal disease. J Clin Periodontol 6:351–382
26. Sokolovsky M, Riordan JF, Vallee BL (1966) Tetranitromethane. A reagent for the nitration of tyrosyl residues in proteins. Biochemistry 5:3582–3589
27. Sveen K, Skaug N (1980) Bone resorption stimulated by lipopolysaccharides from *Bacteroides*, *Fusobacterium* and *Veillonella*, and by the lipid A and the polysaccharide part of *Fusobacterium* lipopolysaccharide. Scand J Dent Res 88:535–542