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Design and Synthesis of Sensitive Fluorogenic Substrates Specific for Lys-Gingipain[1]

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Lys-gingipain (Kgp) is a major cysteine proteinase produced by the oral anaerobic bacterium *Porphyromonas gingivalis*, and has been implicated as a major pathogen in the development and progression of advanced adult periodontitis. This enzyme is believed to act as a major virulence factor of the disease, yet there exist no convenient and sensitive substrates for analyzing its biological activity. For a better understanding of the importance of this enzyme in the organism, there is an urgent need for specific substrates. Here we designed and synthesized two peptide 4-methyl-coumaryl-7-amides (MCA), carbobenzoxy (Z)-His-Glu-Lys-MCA, and Z-Glu-Lys-MCA, and tested their possible use as sensitive substrates for Kgp with limited specificity. Both substrates exhibited greater k_{cat}/K_m values than the best known Kgp substrates described so far. Both substrates were resistant to Arg-gingipain, another pathogenic cysteine proteinase from *P. gingivalis*, as well as trypsin and cathepsins B, L, and H. The levels of Kgp in various microorganisms and human cells were determined with Z-His-Glu-Lys-MCA. Little or no Kgp-like activity was detected in either other microorganisms or human cells tested. These results indicate that the present substrates are a valuable and fast tool for routine assays and for mechanistic studies on Kgp.

Key words: cysteine proteinase, fluorogenic substrate, Lys-gingipain, periodontitis, *Porphyromonas gingivalis*.

Arg-gingipain (Rgp) and Lys-gingipain (Kgp) are two major cysteine proteinases produced in both cell-associated and secretory forms by the Gram-negative, black-pigmented anaerobe *Porphyromonas gingivalis*, and are known to cleave Arg-Xaa and Lys-Xaa peptide bonds, respectively (reviewed in Refs. *1* and *2*). Together, these enzymes are suggested to be responsible for more than 85% of the overall proteolytic activity of the organism (*3*). Recent studies provide evidence indicating that the proteolytic activities of Rgp and Kgp are involved in a wide range of pathologies of adult type periodontitis in terms of destruction of the host periodontal tissues and disruption of host defense mechanisms (*1*, *2*). Moreover, both enzymes are important for providing nutrients for bacterial growth (*4*) and for processing the bacterial cell surface and secretory proteins (*5*, *6*). More recently, we constructed and analyzed Kgp-deficient mutants, thereby providing evidence suggesting that this enzyme is involved in hemagglutination, hemoglobin binding and heme accumulation by the organism, and the bleeding tendency in periodontal pockets (*7*).

Kgp is thus believed to act as a major virulence factor of the organism, yet there exist no convenient and sensitive substrates for its determination. The commercially available peptide substrates so far used, such as *t*-butyloxycarbonyl (Boc)-Val-Leu-Lys-MCA (*8*), *N*-*p*-tosyl-Gly-Pro-Lys-*p*-nitroanilide (pNA) (*9*, *10*), H-Val-Leu-Lys-pNA (*11*), and Z-Lys-pNA (*3*), are not sufficiently sensitive compared with commercially available Rgp substrates such as Z-Phe-Arg-MCA and Boc-Phe-Ser-Arg-MCA. In addition, these substrates appear to be restricted in their selectivity. Therefore, to better understand the pathophysiological roles of Kgp, there is an urgent need to develop better selective and sensitive substrates for this enzyme.

In this paper, we describe the design, synthesis, catalytic properties, and kinetic evaluation of two new fluorogenic substrates suitable for assessing the activity of Kgp. These substrates could be also used to analyze the distribution of Kgp in various microorganisms and host cells.

EXPERIMENTAL PROCEDURES

Peptide Substrates—The two fluorogenic substrates Z-His-Glu-Lys-MCA and Z-Glu-Lys-MCA were synthesized by the usual solution phase protocols in which isobutyl chloroformate/triethylamine or 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide/1-hydroxybenzotriazole was used as a condensing agent. The Lys, Glu, and His side chains were protected by Boc, *tert*-butyl and trityl groups, respectively. The reaction intermediates were purified by silica gel chromatography when necessary. Deprotection was performed by hydrogenation on palladium followed by treatment with trifluoroacetic acid and anisole. The crude substrates were

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purified by reverse-phase chromatography on MCI CHP-20P (Mitsubishi Kasei) using 0.5% HCl and acetonitrile as the eluting solvent.

Z-His-Glu-Lys-MCA: mp. 166–169°C (decomp.); ^{1}H-NMR (CD_3OD) δ 7.93 (s, 1H), 7.65–7.58 (m, 3H), 7.30–7.25 (m, 5H), 6.91 (s, 1H), 6.21 (d, J = 1.2 Hz, 1H), 5.08 (d, J = 12.4 Hz, 1H), 5.04 (d, J = 12.4 Hz, 1H), 4.52–4.48 (m, 1H), 4.35–4.26 (m, 2H), 3.15–3.01 (m, 1H), 2.94–2.90 (m, 2H), 2.43 (s, 3H), 2.38–2.26 (m, 2H), 2.14–2.02 (m, 3H), 1.89–1.81 (m, 1H), 1.74–1.46 (m, 4H); MS (FAB) 704 (MH^+).

Z-Glu-Lys-MCA: mp. 152–156°C (decomp.); ^{1}H-NMR (CD_3OD) δ 7.85 (s, 1H), 7.65 (d, J = 8 Hz, 1H), 7.56 (d, J = 8 Hz, 1H), 7.35–7.24 (m, 5H), 6.21 (s, 1H), 5.13 (d, J = 12.4 Hz, 1H), 5.08 (d, J = 12.4 Hz, 1H), 4.55–4.51 (m, 1H), 4.16–4.12 (m, 1H), 2.95–2.91 (m, 2H), 2.43 (s, 3H), 2.35–2.29 (m, 2H), 2.11–1.98 (m, 3H), 1.86–1.79 (m, 1H), 1.76–1.62 (m, 2H), 1.59–1.45 (m, 2H), MS (FAB) 567 (MH^+).

These substrates were dissolved in 10% dimethylsulfoxide at 10 mM and stored at –20°C until use.

Enzymes—Kgp was purified from the culture supernatant of an Rgp-deficient mutant of *P. gingivalis* (KDP112) according to the previously described method (*8*). Rgp (*12*) and cathepsins B (*13*), L (*14*), and H (*15*) were purified as described. All other chemicals were of reagent grade and were purchased from various commercial sources.

Assays—Kgp activity was determined spectrophotometrically with the two substrates at a variety of pH values. Reaction mixtures contained 200 μl of buffer (for routine assays, 0.1 M sodium phosphate buffer, pH 7.5, was used), 100 μl of 50 mM cysteine, 500 μl of substrate solution, and 200 μl of sample solution in a total volume of 1 ml. The final substrate concentration range was 5–20 μM; the final dimethylsulfoxide concentration was 0.005% for all assays. Reaction mixtures were incubated at 40°C for 10 min and the reaction was terminated by adding 1 ml of 10 mM iodoacetic acid in 0.1 M sodium acetate buffer (pH 5.0). The released 7-amino-4-methyl-coumarin (AMC) was measured at 460 nm (excitation at 380 nm) using a fluorescence spectrophotometer. One unit of enzyme activity was defined as the amount of enzyme required to release 1 nmol of 7-amino-4-methylcoumarin/ml under these conditions. Kinetic parameters were obtained with appropriate concentrations of substrates. In all cases, the kinetics of cleavage followed the Michaelis-Menten scheme. The K_m and V_{max} values were estimated from the intercepts and slopes of double-reciprocal plots of rate versus substrate concentration. The k_{cat} values (s^{-1}) were determined from the equation

$$k_{cat} = \frac{V_{max}}{\Delta A/[S]\times[E]}$$

where ΔA, [S], and [E] are the increase in absorbance, substrate concentration (μM), and enzyme concentration (molar), respectively. The active enzyme concentration was determined by titration against a competitive Kgp-specific inhibitor (not shown). Cathepsins B and L were assayed with Z-Phe-Arg-MCA (*13*, *14*) and cathepsin H was assayed with Arg-MCA (*15*).

Bacterial Strains and Culture Conditions—Various *P. gingivalis* mutants, such as a Kgp-deficient mutant (KDP-129) (*4*, *7*), an Rgp-deficient mutant (KDP133) (*4*, *16*) and an Rgp and Kgp-deficient mutant (KDP136) (*4*), were constructed as previously described. Besides these mutants, various bacterial strains were maintained on blood agar plates and grown in enriched brain heart infusion broth containing hemin (5 μg/ml) and menadione (0.5 μg/ml) under anaerobic conditions (10% CO_2, 10% H_2, 80% N_2) (*4*).

RESULTS AND DISCUSSION

It has previously been shown that Kgp exhibits little or no activity toward various synthetic substrates, such as Z-Phe-Arg-MCA (a substrate for Rgp and cathepsins B and L), Boc-Phe-Ser-Arg-MCA (a substrate for Rgp and plasmin), Suc-Leu-Leu-Val-Tyr-MCA and Suc-Ala-Ala-Pro-Phe-MCA (for chymotrypsin), and Suc-Ala-Pro-Ala-MCA (a substrate for elastase) (*8*). In addition, Kgp is known to have no detectable aminopeptidase activity. Based on these data, the enzyme was suggested to show a strong preference for substrates containing Lys in the P1 site. Kgp was also shown to be capable of hydrolyzing protein substrates, such as bovine hemoglobin, bovine serum albumin, casein, and types I and IV collagen (*8*). More recently, we found that human salivary histatins were a better substrate for both Rgp and Kgp. Histatins comprise a family of histidine-rich polypeptides having antibacterial and antifungal activity (*17*, *18*) and histamine-releasing activity in mast cells (*19*). Histatins 1, 3, and 5 are major histatins in saliva (*20*) and are mutually homologous. Thus the cleavage-sites of these histatins by Kgp were determined by reversed-phase high-performance liquid chromatography followed by NH_2-terminal amino acid sequencing as described (*20*). All of the hist-

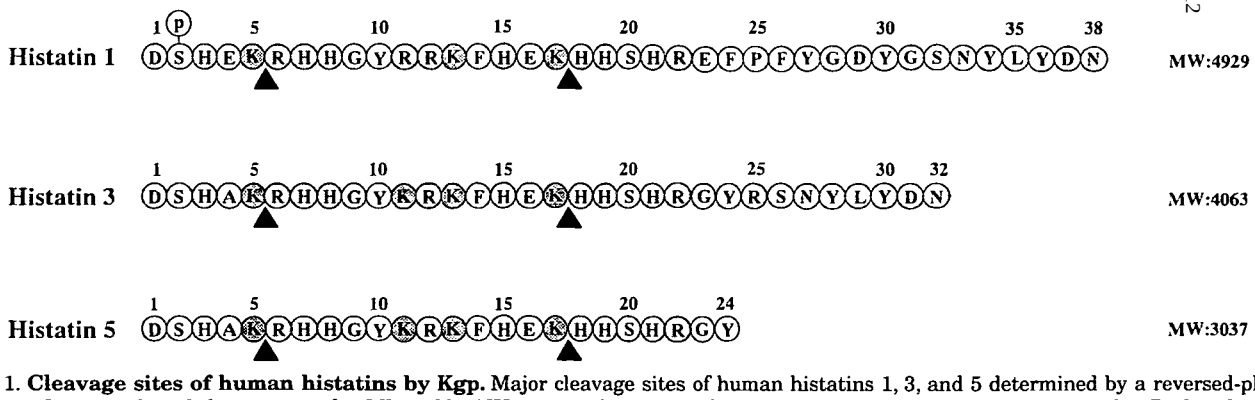

Fig. 1. **Cleavage sites of human histatins by Kgp.** Major cleavage sites of human histatins 1, 3, and 5 determined by a reversed-phase high-performance liquid chromatography followed by NH_2-terminal amino acid sequencing are indicated by closed triangles. P, phosphorylation.

atins were cleaved most efficiently at Lys[5]-Arg[6] and Lys[17]-His[18] bonds (Fig. 1), suggesting that Kgp exhibits one of the most restricted specificities known among cysteine proteinase family members. Basic amino acid residues in the P1′ positions of these histatins appear to be important for the cleavage specificity of Kgp. A His residue in the P3 position appears to be preferred by Kgp. We therefore designed and synthesized two peptide substrates, Z-His-Glu-Lys-MCA and Z-Glu-Lys-MCA.

Our previous study demonstrated that the optimal pH for the hydrolysis of the best commercially available substrate for Kgp, Boc-Val-Leu-Lys-MCA, is around 7.5, and that the enzyme exhibits relatively high activity even at pH values between 6.5 and 7.0 and between 8.0 and 9.5 (*8*). When the present substrates were incubated with Kgp in a wide range of buffers (pH 4.0–11.0) at 40°C for 10 min, single peaks were observed for each substrate (Fig. 2). The optimal pH was found to be around 8.0 for each substrate. At pH 7.5, Kgp showed more than 85% of the maximal activity obtained at pH 8.0. At pH 8.5, the enzyme retained more than 90% of the maximal activity for both Z-His-Glu-Lys-MCA and Z-Glu-Lys-MCA. Kgp hydrolyzed both substrates five to six times more efficiently than did the previous substrate, Boc-Val-Leu-Lys-MCA (about 570% for Z-His-Glu-Lys-MCA, about 530% for Z-Glu-Lys-MCA). The cleavage rates of both substrates by other proteinases, such as Rgp, cathepsins B, L and H, and trypsin, were determined at their respective optimal pH values (Table I). All of these enzymes exhibited little or no activity toward these two substrates, indicating that they are selective and sensitive substrates for Kgp.

The fluorescence spectrophotometer was set to read fluorescence intensity between 1 and 100, where the reading is

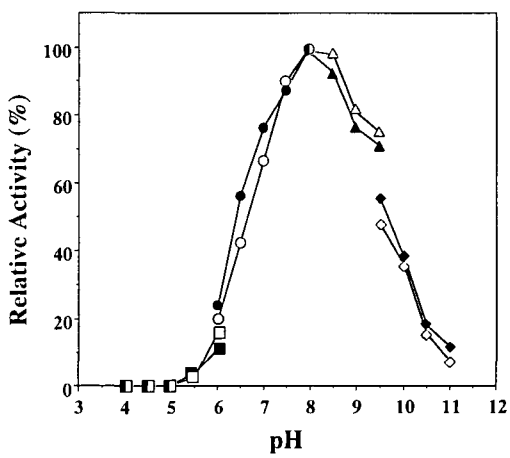

Fig. 2. **pH dependence of the hydrolysis of Z-His-Glu-Lys-MCA and Z-Glu-Lys-MCA by Kgp.** The conditions for the hydrolysis of Z-His-Glu-Lys-MCA (open symbols) and Z-Glu-Lys-MCA (closed symbols) are described in "EXPERIMENTAL PROCEDURES." The buffers used were 0.1 M sodium acetate (pH 4.0–6.0, squares), 0.02 M sodium phosphate (pH 6.0–8.0, circles), 0.02 M sodium barbital (pH 8.0–9.5, triangles), and 0.1 M glycine-NaOH (9.5–11.0, diamonds). The point of maximal activity was taken as 100% in each case.

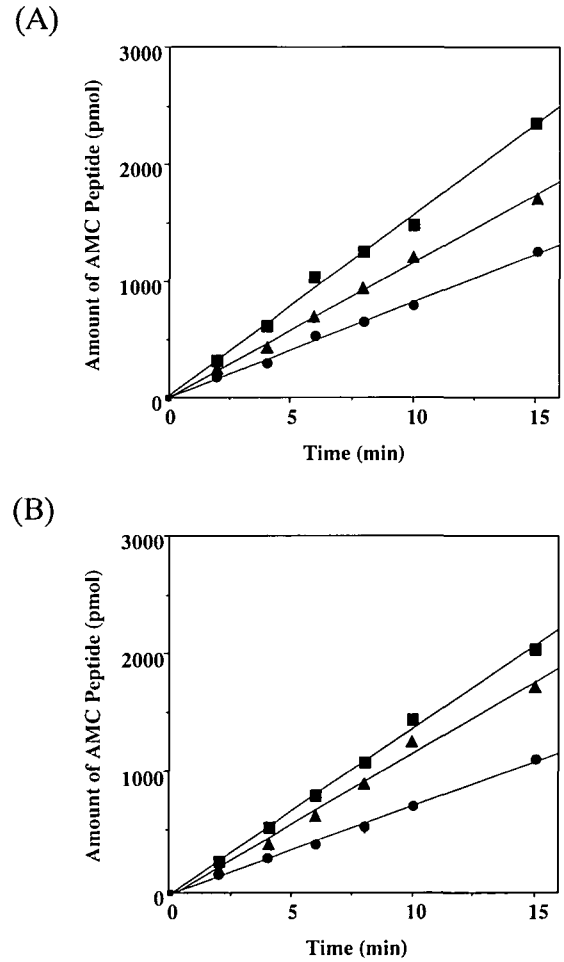

Fig. 3. **Effect of substrate concentration on the hydrolysis of Z-His-Glu-Lys-MCA (A) and Z-Glu-Lys-MCA (B) by Kgp.** Kgp (0.1 μg) was incubated with 5 μM (●), 10 μM (▲), and 20 μM (■) of each substrate at 40°C for the indicated time intervals. Values are the means of at least three experiments.

TABLE I. **Hydrolysis of various fluorogenic synthetic substrates by Kgp, Rgp, cathepsins B, L, and, H, and trypsin.** Comparison of the rates of hydrolysis of Z-His-Glu-Lys-MCA and Z-Glu-Lys-MCA by Kgp, Rgp, cathepsins B, L, and H, and trypsin relative to those of the best substrate for each enzyme. Values are expressed as the percent of the maximal activity of each enzyme determined at its optimal pH value with the best substrate.

Substrate	% of maximum activity					
	Kgp	Rgp	Cathepsin B	Cathepsin L	Cathepsin H	Trypsin
Z-His-Glu-Lys-MCA	100	0	0.9	0.09	0.03	9.1
Z-Glu-Lys-MCA	93	0	3.3	0.28	0.12	0.4
Z-Phe-Arg-MCA	0	98	100	100	n.d	n.d
Arg-MCA	0	3	n.d	n.d	100	n.d
Boc-Phe-Ser-Arg-MCA	0	100	n.d	n.d	n.d	100

n.d, not determined.

considered to be a more reliable indication of the amount of liberated AMC. The cleavage of both substrates by Kgp was linear with enzyme concentration. Figure 3 shows the effects of substrate concentration and incubation time on Kgp activity. Kgp appeared not to be saturated for at least 15 min when more than 5 μM of each substrate (5–200 μM) was used. The use of 5–20 μM of these substrates brought the assay for Kgp into the sensitivity range of 0.1–7.2 μg of enzyme. In the standard procedure, therefore, 20 μM substrate was used.

The kinetic parameters determined for the hydrolysis of both substrates by Kgp are shown in Table II. Lineweaver-Burk plots for the hydrolysis of Z-His-Glu-Lys-MCA and Z-Glu-Lys-MCA by Kgp gave k_{cat}/K_m values of 1779 and 1867 $M^{-1}\cdot s^{-1}$, respectively. The data show the two substrates to have greater k_{cat}/K_m values than the best substrates for Kgp described so far. There was a nearly 20-fold increase in the k_{cat}/K_m value of Kgp with the present substrates in comparison with the previous substrate, Boc-Val-Leu-Lys-MCA. Although the presence of His at the P3 position appears to be important for the cleavage of histatins, it was not necessarily essential for the enzyme-synthetic substrate interaction with efficient cleavage by Kgp.

In order to determine whether Kgp is characteristic of strains of *P. gingivalis*, we examined the distribution among other microorganisms and host cells using Z-His-Glu-Lys-MCA as a substrate. Table III shows a comparison of the rates of hydrolysis of the substrate by culture supernatants and cell extracts of various microorganisms. The rates are expressed as percentage of the activity (units/mg protein) determined in *P. gingivalis* ATCC33277. The enzyme was found ubiquitously but unevenly in both culture supernatants and cell extracts of all the *P. gingivalis* strains examined, including ATCC33277, 381, W50, and SU63 (not shown). The Rgp-deficient mutant (KDP133) also showed a significant Kgp activity in both the culture supernatant and cell extracts. However, the Kgp activity was not detectable in either the culture supernatants or the cell extracts of the Kgp-deficient mutant (KDP129) and the Rgp and Kgp-deficient mutant (KDP136) of *P. gingivalis*. Other oral bacteria, including *Bacteroides forsythus, Fusobacterium nucleatum, Prevotella intermedia, Prevotella melaninogenica, Prevotella denticola, Actinomyces viscosus*, and *Actinobacillus actinomycetemcomitans* showed no hydrolyzing activity for this substrate, suggesting that a Kgp-like enzyme is not contained in these microorganisms. The distribution of Kgp in these organisms is very similar to that of Rgp, as revealed by assay using Z-Phe-Arg-MCA as a substrate. These data strongly suggest that not only Rgp, but also Kgp is characteristically produced by *P. gingivalis* (Table III). In addition, the Kgp activity determined with Z-His-Glu-Lys-MCA was not detectable in human mononuclear, polymorphonuclear leukocytes, gingival fibroblasts or plasma (Table IV). On the other hand, significant enzyme activity, as revealed by assay with Z-Phe-Arg-MCA, was

TABLE II. **Kinetic parameters for the hydrolysis of fluorogenic substrates by Kgp.** All mesurements were carried out at pH 8.0 in 10 mM sodium phosphate buffer at 40°C for 10 min.

Substrate	$k_{cat} \times 10^{-3}$ (s^{-1})	K_m (μM)	k_{cat}/K_m ($M^{-1}\cdot s^{-1}$)
Boc-Val-Leu-Lys-MCA	18.1	166.6	109
Z-His-Glu-Lys-MCA	25.8	14.5	1779
Z-Glu-Lys-MCA	26.7	14.3	1867

TABLE IV. **Distribution of the proteolytic activities of Rgp and Kgp determined with Z-Phe-Arg-MCA and Z-His-Glu-Lys-MCA as substrates, respectively, in human mononuclear and polymorphonuclear leukocytes, gingival fibroblasts, and plasma.** Freshly prepared mononuclear and polymorphonuclear leukocytes and plasma from human donors and a human gingival fibroblast cell line (Gin-1) were suspended in phosphate-buffered saline containing 0.05% Brij 35. Other details are the same as described in Table III.

Sources	Proteolytic activity (%)	
	Cell extract	
	Z-Phe-Arg-MCA	Z-His-Glu-Lys-MCA
Porphyromonas gingivalis ATCC 33277	100	100
Human mononuclear leukocyte	0.65	0.33
Human polymorphonuclear leukocyte	1.41	0.14
Human plasma	0.0008	0.0009
Human gingival fibroblast	2.84	0

TABLE III. **Distribution of the proteolytic activities of Rgp and Kgp determined with Z-Phe-Arg-MCA and Z-His-Glu-Lys-MCA as substrates, respectively, in both culture supernatants and cell extracts of various microorganisms.** The culture supernatants of various microorganisms were separated by centrifugation (12,000 ×*g* for 20 min) and then subjected to ammonium sulfate precipitation. The precipitates were resuspended in phosphate-buffered saline and dialyzed against the same buffer After removal of the culture supernatants, the pellets were suspended in phosphate-buffered saline containing 0.05% Brij 35, sonicated and then centrifuged at 105,000 ×*g* for 30 min (cell extracts). Values are expressed as the relative ratio of each enzyme activity (units/mg protein) in the fractions from various microorganisms to that from the wild-type *P. gingivalis* ATCC 33277.

Sources		Proteolytic activity (%)			
		Z-Phe-Arg-MCA		Z-His-Glu-Lys-MCA	
		Culture supernatant	Cell extract	Culture supernatant	Cell extract
Porphyromonas gingivalis	ATCC 33277	100	100	100	100
	KDP 129	102.0	44.3	0.016	0.024
	KDP 133	0	0.063	31.5	6.47
	KDP 136	0	0.005	0	0.004
Bacteroides forsythus	ATCC 43037	0.005	1.23	0.0001	0.11
Fusobacterium nucleatum	ATCC 23726	0.001	0.004	0.004	0.022
Prevotella intermedia	ATCC 25611	0.001	0	0.007	0.019
	ATCC 33563	0.007	0	0.007	0
Prevotella melaninogenica	ATCC 25845	0.010	0	0.004	0.015
Prevotella denticola	ATCC 33185	0.004	0.015	0.004	0.015
Actinomyces viscosus	NY-1	0.001	0.022	0	0.032
Actinobacillus actinomycetemcomitans	JP2	0	0.0002	0.001	0.016

detected in these cells, which was probably due to cathepsins B, L, and kallikrein derived from the host cells. The extremely low level of Z-Phe-Arg-MCA-hydrolyzing activity in human plasma appears to be due to plasma proteinase inhibitors such as kininogen.

In conclusion, Z-His-Glu-Lys-MCA and Z-Glu-Lys-MCA are the most sensitive and selective Kgp substrate described so far. The k_{cat}/K_m value for Kgp is higher than those reported previously. A simple assay method using these substrates is suitable for the assessment of Kgp activity in crude preparations.

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