Oral Microbiology Immunology 2005: 20: 67–72
Printed in Singapore. All rights reserved

Oral Microbiology and Immunology

Isolation and characterization of aminopeptidase from *Capnocytophaga granulosa* ATCC 51502

K. Ohishi, T. Yamamoto, T. Tomofuji, N. Tamaki, T. Watanabe
Department of Oral Health, Okayama University Graduate School of Medicine and Dentistry, Okayama, Japan

Ohishi K, Yamamoto T, Tomofuji T, Tamaki N, Watanabe T. Isolation and characterization of aminopeptidase from Capnocytophaga granulosa *ATCC 51502.*
Oral Microbiol Immunol 2005: 20: 67–72. © Blackwell Munksgaard, 2005.

There is evidence that enzymes from the genus *Capnocytophaga* play a role in dental calculus formation. Although most of the species in the genus produce aminopeptidases, there is a paucity of data on the purification and characterization of the enzyme, except in the case of *Capnocytophaga gingivalis*. The aim of this study was to purify aminopeptidase from culture supernatant of *Capnocytophaga granulosa* ATCC 51502, a new species of the genus. Purification was performed using ammonium sulfate fractionation and two chromatographic steps. The aminopeptidase was purified 158,433-fold with a yield of 12.0%. The enzyme appeared to be a trimer with a molecular mass of 270 kDa. The optimal pH of the aminopeptidase was 6.5 and its activity was completely inhibited by incubation at 50°C for 10 min. The enzyme showed maximum specificity for basic amino acids (Arg and Lys) and also hydrolyzed noncharged amino acids (Met, Leu and Ala). Ca^{2+}, Zn^{2+} and Fe^{3+} activated the enzyme, while EDTA, Ag^{+}, Hg^{+} and Cu^{2+} inhibited it. These results suggest that aminopeptidase of *C. granulosa* is different from that of *C. gingivalis* but similar to aminopeptidase B.

Key words: aminopeptidase; arginine; *Capnocytophaga granulosa*; dental calculus

Tatsuo Watanabe, Department of Oral Health, Okayama University Graduate School of Medicine and Dentistry, 2-5-1 Shikata-cho, Okayama 700–8525, Japan
Tel.: +81 86 235 6710;
fax: +81 86 235 6714;
e-mail: wyobou@md.okayama-u.ac.jp
Accepted for publication July 28, 2004

There has been some evidence that bacterial proteases play a role in dental calculus formation. Calculus level is positively correlated with protease activity in human saliva (37), with the protease believed to originate from bacteria (36). Supragingival plaque from calculus formers has been reported to exhibit significantly higher protease activity than that from noncalculus formers (23). Finally, bacterial protease from *Bacteroides loescheii* degrades salivary inhibitors of calcium phosphate precipitation *in vitro* (22).

There is also evidence for a specific role of *Capnocytophaga* species in calculus formation as follows. Both the proportion and prevalence of *Capnocytophaga* species are higher in plaque samples from calculus formers than in samples from noncalculus formers (25) and the species exhibits high aminopeptidase activity (17, 28, 34, 35). Moreover, a bacterial cell suspension of *Capnocytophaga gingivalis* has been demonstrated to destroy calcium phosphate precipitation inhibitors in saliva *in vitro* (24).

Capnocytophaga granulosa is a newly isolated species from human dental plaque (38). This species is facultative anaerobic, and exhibits high aminopeptidase activity and granular inclusions in its cells. Although aminopeptidase activity has been reported upon, there are few reports of the isolation and characterization of aminopeptidase from *Capnocytophaga* species (33). The aim of this study was to isolate and characterize aminopeptidase from culture supernatant of *C. granulosa*.

Materials and methods

Bacterial strain and growth conditions

C. granulosa ATCC 51502 was cultured at 37°C for 48 h in an anaerobic chamber (85% N_2, 5% CO_2, 10% H_2) in Gifu anaerobic medium (GAM) broth (Nissui Seiyaku, Tokyo, Japan).

Purification of aminopeptidase

Extraction of the crude enzyme and all purification procedures were performed at 4°C. The culture fluid (volume 62.7 liters) was obtained by centrifugation of the whole

culture (12,000 × *g*, 10 min, 4°C). Ammonium sulfate was added to the culture supernatant to 60% saturation. The precipitated proteins were resuspended in 20 mM phosphate buffer, pH 7.0. After dialysis against the same buffer at 4°C for 18 h, insoluble materials were removed by centrifugation at 15,000 × *g* for 20 min. Extraction of the enzyme from the insoluble materials was repeated four times using the same buffer. The proteins in the dialysate and the extracted protein were subsequently mixed and concentrated by ultrafiltration (Ultrafilter YM-10, cut off 10,000, Millipore Co., Bedford, MA). The concentrate was subsequently dialyzed against 10 mM phosphate buffer for 12 h and then adsorbed to a CM Sepharose Fast Flow column (5 × 14.5 cm; Amersham Biosciences Co., Piscataway, NJ) that was equilibrated with the same buffer. After washing the column with the buffer, the enzyme was eluted first with 100 mM and then with 200 mM of NaCl in the buffer at a flow rate of 120 ml/h. Active fractions were pooled and concentrated using an Ultrafilter YM-10 and Centriplus YM-100 (cut off 100,000; Millipore Co.). The solution was then applied to a column of Superdex 200 prep grad (1.6 × 54 cm; Amersham Biosciences Co.), equilibrated with 10 mM phosphate buffer (pH 7.0) containing 200 mM NaCl. The enzyme was eluted in the same buffer at a flow rate of 45 ml/h.

Assay of aminopeptidase activity

Aminopeptidase activity was measured using Arg-β naphthylamide (NA) (Sigma Chemical Co., St. Louis, MO) (35). The reaction mixture was prepared by mixing 0.3 ml of 1 mM substrate, 0.45 ml of 100 mM phosphate buffer (pH 6.5), 0.3 ml of distilled water, and an appropriate amount of the enzyme in a volume of 0.15 ml. After incubation for 1 h at 37°C, 0.3 ml of a solution of the stabilized diazonium salt Garnet GBC (0.5 mg/ml, Sigma Chemical Co.) in 1 M acetic acid, buffered at pH 4.2 and containing 10% Tween 20, was added. After the mixture was allowed to stand for 15 min at room temperature, the absorbance was read at 525 nm, using a spectrophotometer (UV-1200, Shimadzu Co., Kyoto, Japan). Enzyme activities were expressed as nanomoles of β-naphthylamine liberated in 1 min at 37°C under the conditions described above.

Protein determination

Protein was determined using the method of Lowry et al. (19) modified by Hartree (10) or the method of Böhlen et al. (4). Bovine serum albumin (Sigma Chemical Co.) was used as standard.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE was performed according to the method of Laemmli (16), using a mini-gel apparatus (KS-8010, Marisol Co., Tokyo, Japan) and 10% separating gels. Samples were dissolved in sample buffer containing 2% SDS, and then incubated at 100°C for 3 min. A mixture of three protein standards (LMW electrophoresis calibration kit; Amersham Biosciences Co.) was electrophoresed in parallel. Electrophoresis was performed at 20 mA for 90 min and the gel was stained with 2D-silver stain reagents II, 'Daiichi' (Daiichi Pure Chemicals Co., Tokyo, Japan).

Molecular mass determination

The molecular mass of the native enzyme was estimated by gel filtration (2) using a Superdex 200 prep grad column (1.6 × 54 cm; Amersham Biosciences Co.), as described above. The column was calibrated using the following standard proteins: thyroglobulin (669 kDa), apoferritin (443 kDa), alcohol dehydrogenase (150 kDa), bovine serum albumin (66 kDa) and carbonic anhydrase (29 kDa) (Sigma Chemical Co.). The molecular mass of the enzyme under denaturing conditions was also determined by SDS-PAGE, as described above.

Determination of isoelectric point

The isoelectric point was determined using an IEF PAGE mini pH 3–10 (Tefco International Co., Tokyo, Japan) and IEF pH 3–10 buffer kit (Tefco International Co.). After applying the purified enzyme, electrophoresis was performed at a voltage of 100 V for 1 h, followed by 200 V for 1 h and 500 V for 30 min. A mixture of 12 protein markers with isoelectric points ranging from 3.50 to 9.30 (Amersham Biosciences Co.) was used to estimate the isoelectric point. The gel was stained with 2D-silver stain reagents II, 'Daiichi'.

Activity staining was performed using the method of Mort & Leduc (26). The electrophoresed gel was incubated in the mixture of 3 ml of 1 mM L-Arg-βNA, 4.5 ml of 100 mM phosphate buffer (pH 6.5), and 3 ml of distilled water. After removing the reaction mixture, 0.5 mg/ml diazonium salt Garnet GBC and 1 M acetic acid buffer (pH 4.2) containing 10% Tween 20 were added for 5 min and then washed off with water.

Effect of pH and temperature

The effect of pH on aminopeptidase activity was examined using 100 mM phosphate buffer at pH values ranging from 6.0 to 8.0. The pH stability of the aminopeptidase was tested by incubation of the purified enzyme (60 ng) for 30 min at 0°C. The results were expressed as a percentage of the activity obtained at optimum pH.

The temperature stability was tested by incubation of the enzyme at 0, 25, 37 and 50°C for 10, 30, 60 and 120 min in 100 mM phosphate buffer, pH 6.5, followed by the standard enzyme activity assay.

Substrate specificity

The relative activities of the aminopeptidase from *C. granulosa* against Arg-βNA, Lys-βNA, Met-βNA, Leu-βNA, Ala-βNA, Glu-βNA, Ser-βNA, Tyr-βNA, benzoyl-Arg-βNA, Phe-Phe-Ala-βNA, Leu-Gly-Gly-4-methoxy-βNA and Gly-Pro-Leu-βNA (Sigma Chemical Co.) were determined using a standard activity assay. The K_m and V_{max} were obtained from a Michaelis–Menten plot.

Effect of protease inhibitors and metal cations

Protease inhibitors such as phenylmethylsulfonyl fluoride (PMSF), 4-(2-aminoethyl)-benzenesulfonyl fluoride (AEBSF), antipain, leupeptin, tosyl-L-lysine chloromethyl ketone (TLCK), tosyl-L-phenylalanine chloromethyl ketone (TPCK), chymostatin, iodoacetate, N-ethylmaleimide, *trans*-epoxysuccinyl-L-leucylamido-(4-guanidino) butane (E-64), pepstatin and EDTA, and metal cations such as $AgNO_3$, $HgCl_2$, $CuSO_4$, $MgCl_2$, $CaCl_2$, $ZnCl_2$ and $FeCl_3$, were added to separate enzyme solutions. The purified enzyme was incubated in the presence of each inhibitor for 30 min and followed by the standard enzyme assay with Arg-βNA as the substrate. Activity was expressed as a percentage of the activity obtained in the absence of the added inhibitor.

Amino acid analysis

The purified enzymes were hydrolyzed with 6 N HCl at 110°C for 24 h in a sealed and evacuated tube, and the amino acid compositions were determined with an automatic amino acid analyzer (Model

L-8500, Hitachi Ltd, Tokyo, Japan) (32). For analysis of cysteine, the purified enzymes were oxidized with performic acid before the hydrolyzation (11). Tryptophan was analyzed after hydrolyzation with 4 M methanesulfonic acid containing 0.2% tryptamine (18).

Results

Purification of aminopeptidase

Aminopeptidase was purified from the cell culture supernatant of *C. granulosa* ATCC 51502 by sequential column chromatography. Figures 1 and 2 show the chromatographic profiles of the enzyme on CM Sepharose Fast Flow (4th step) and gel filtration on Superdex 200 prep grad (final step), respectively. A summary of the purification steps is provided in Table 1. The final enzyme sample was purified 158,433-fold with a 12.0% yield.

Purity and molecular mass of aminopeptidase

The fractions from each purification step with enzyme activity were analyzed by SDS-PAGE under unheated conditions (Fig. 3). The final preparation showed a single band with a molecular mass of 86 kDa (lane 6).

The relative molecular mass of the native enzyme estimated by gel filtration on a Superdex 200 prep grad column was around 270 kDa.

Isoelectric point determination

The isoelectric points were estimated to be 7.5, 8.7, 8.8 and 8.9. Bands with aminopeptidase activity were observed around isoelectric points 8.8 and 7.5.

Effects of pH and temperature

With 100 mM phosphate buffer and 1 mM Arg-βNA, the optimum pH of the purified enzyme was 6.5 (Fig. 4). The enzyme was stable in the pH range 6.6–7.0. Heating the enzyme at 50°C for 10 min resulted in complete loss of activity.

Substrate specificity

The substrate specificity is shown in Table 2. The purified enzyme was active against Arg-βNA, Lys-βNA, Met-βNA, Leu-βNA and Ala-βNA. The enzyme was more specific for substrates containing basic amino acids such as lysine and arginine at the first positions. The enzyme did not hydrolyze benzoyl-Arg-βNA and substrates for endopeptidase.

Effects of protease inhibitors and metal cations

The influence of protease inhibitors on enzyme activity is shown in Table 3. The

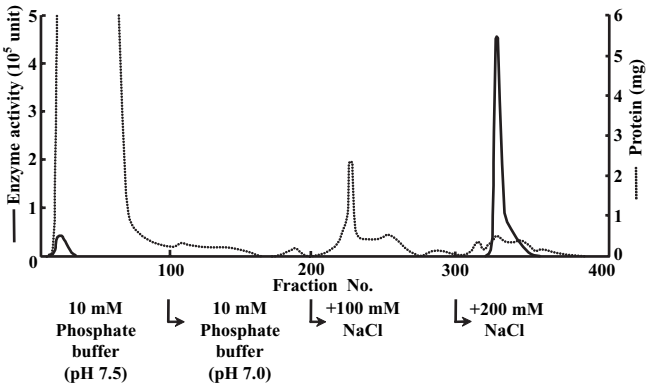

Fig. 1. Cation-exchange chromatography of the filtered sample after ammonium sulfate fractionation using a CM Sepharose Fast Flow column.

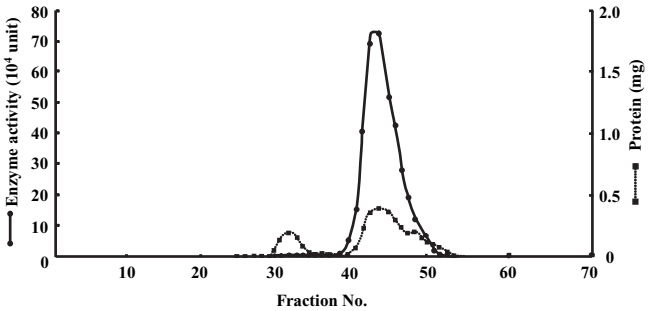

Fig. 2. Gel filtration profile of the fraction after the 2nd ultrafiltration on Superdex 200 prep grad column.

Table 1. Summary of purification steps of aminopeptidase from *Capnocytophaga granulosa* ATCC 51502

Purification step	Total protein (mg)	Total activity (10^5 unit)	Specific activity (unit/mg)	Purification (Fold)	Yield (%)
Culture supernatant	1,862,019	35.0	1.88	1	100
$(NH_4)_2SO_4$ precipitate	19,415	19.7	101	54	56.3
Ultrafilter YM-10 (1st)	14,435	17.8	123	65	50.9
CM Sepharose Fast Flow	17.9	9.9	55,307	29,419	28.3
Ultrafilter YM-10 (2nd)	7.5	6.8	90,667	48,227	19.4
Centriplus YM-100	4.1	5.1	124,390	66,165	14.6
Superdex 200 prep grad	1.41	4.2	297,872	158,443	12.0

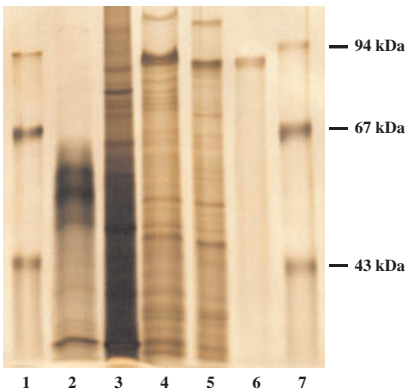

Fig. 3. SDS-PAGE of aminopeptidase from *C. granulosa* ATCC 51502 at various stages of purification on 10% separating gel stained with 2D-silver stain reagents II. Lanes: 1 and 7, high molecular-size standards; 2, crude culture supernatant; 3, fraction after ammonium sulfate precipitation; 4, active enzyme fraction from CM Sepharose Fast Flow chromatography; 5, fraction after 2nd ultrafiltration; and 6, purified enzyme fraction from Superdex 200 prep grad chromatography.

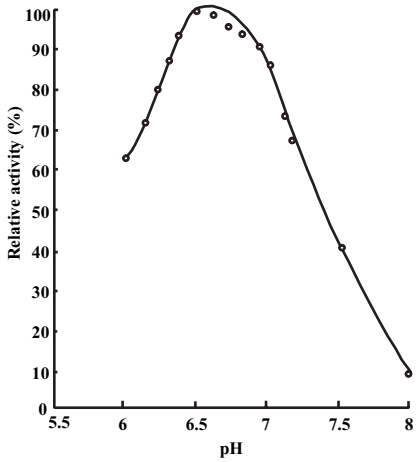

Fig. 4. Effect of pH on the activity of the purified enzyme against Arg-βNA. Assay mixture adjusted to various pHs with 100 mM phosphate buffer.

Table 2. Substrate specificity of aminopeptidase from *Capnocytophaga granulosa* ATCC 51502

Substrate	K_m (mM)	V_{max} (nmol/min)
Arg-βNA	0.046	3.27×10^4
Lys-βNA	0.054	4.30×10^4
Met-βNA	0.111	2.10×10^4
Leu-βNA	0.148	5.40×10^4
Ala-βNA	0.232	0.73×10^4
Glu-βNA	– [a]	–
Ser-βNA	–	–
Tyr-βNA	–	–
Benzoyl-Arg-βNA	–	–
Phe-Phe-Ala-βNA	–	–
Leu-Gly-Gly-4-methoxy-βNA	–	–
Gly-Pro-Leu-βNA	–	–

[a] – = Not detected.

chelating agent EDTA inhibited 86% of enzyme activity, while E-64, an inhibitor of cysteine proteinases, inhibited 16% of activity. Serine-protease inhibitors such as PMSF, AEBSF, antipain, leupeptin, TLCK, TPCK and chymostatin, sulfhydryl group reagents such as iodoacetate and N-ethylmaleimide, and an aspartate-specific inhibitor, pepstatin, had no effect on enzyme activity.

The effects of various cations were also examined (Table 4). Ag^+, Hg^{2+}, and Cu^{2+} inhibited enzyme activity by 100%, 100% and 84%, respectively. In contrast, Ca^{2+}, Zn^{2+} and Fe^{3+} had a stimulatory effect (26–34%) on enzyme activity.

Amino acid composition

Table 5 summarizes the data obtained from amino acid analyses of the purified enzyme. Glutamic acid, lysine, leucine, alanine and aspartic acid were present more frequently than other amino acids.

Table 3. Effect of various protease inhibitors on aminopeptidase from *Capnocytophaga granulosa* ATCC 51502

Inhibitors[a]	Relative activity (%)
None	100
PMSF	96
AEBSF	96
Antipain	103
Leupeptin	103
TLCK	96
TPCK	95
Chymostatin	98
Iodoacetate	101
N-ethylmaleimide	98
E-64	84
Pepstatin	104
EDTA	14

[a]Concentration, 0.1 mM; PMSF, phenylmethylsulfonyl fluoride, AEBSF, 4-(2-aminoethyl)-benzenesulfonyl fluoride; TLCK, tosyl-L-lysine chloromethyl ketone; TPCK, tosyl-L-phenylalanine chloromethyl ketone; E-64, *trans*-epoxysuccinyl-L-leucylamido-(4-guanidino) butane.

Table 4. Effect of heavy metals on aminopeptidase activity from *Capnocytophaga granulosa* ATCC 51502

Metals[a]	Relative activity (%)
None	100
$AgNO_3$	0
$HgCl_2$	0
$CuSO_4$	16
$MgCl_2$	98
$CaCl_2$	126
$ZnCl_2$	134
$FeCl_3$	129

[a]Concentration, 0.1 mM.

Table 5. Amino acid composition of aminopeptidase from *Capnocytophaga granulosa* ATCC 51502

Amino acid	Composition given in residues in 1000
Asp	76
Thr	45
Ser	50
Pro	44
Glu	110
Gly	69
Ala	80
Met	14
Val	59
Ile	46
Leu	88
Phe	49
Lys	92
His	16
Tyr	66
Arg	37
Trp	56

Discussion

In the current study, the enzyme in the culture supernatant of *C. granulosa* ATCC 51502 was purified and characterized. This enzyme is classified as a metallopeptidase (3). While inhibitors for serine protease, sulfhydryl group reagents, and aspartate-specific inhibitors had no effect on the activity of the enzyme, it was inhibited by the chelating agent. Conversely, addition of Ca^{2+}, Zn^{2+} and Fe^{3+} increased enzyme activity. The enzyme is also classified as an exopeptidase because it exclusively released N-terminal amino acid residues but did not hydrolyze benzoyl-Arg-βNA.

The enzyme differs from those purified from *Capnocytophaga* species in other studies. Enzymes in the envelope fraction of *C. gingivalis* hydrolyze synthetic substrate for chymotrypsin or trypsin (31), neither of which was hydrolyzed by the enzyme purified in this study. The molecular mass of *C. gingivalis* aminopeptidase has been reported to be 64 kDa (33), smaller than the purified enzyme in the current study (86 kDa). The isoelectric point of *C. gingivalis* aminopeptidase (6.3) is lower than that of *C. granulosa* (8.9, 8.8, 8.7, and 7.5). Moreover, the *C. gingivalis* aminopeptidase hydrolyzes Glu-βNA, Ser-βNA and Tyr-βNA, none of which was hydrolyzed by the enzyme purified in the current study.

The purified enzyme shares some characteristics with aminopeptidase B. Aminopeptidase B, also named arginyl aminopeptidase, is one of the major aminopeptidases in mammal organs such as skeletal muscle (8, 14), placenta (27), liver (12, 13, 15), and the testis (7). Aminopeptidase enzyme hydrolyzes Arg-βNA and Lys-βNA (12, 27), the activity being lost by heating at 50°C (15) and inhibited by EDTA (8, 12, 15, 27). The amino acid composition of the purified enzyme was similar to that of aminopeptidase B (7), although aspartic acid, glutamic acid, lysine, tyrosine and tryptophan were more frequently present in the purified enzyme than in aminopeptidase B.

Arginyl aminopeptidases of bacteria have been previously purified and characterized (5, 9, 30). These enzymes and the enzyme purified in the current study have several common characteristics related to their substrate specificity and inhibitors of activity. They hydrolyze both Arg-βNA and Lys-βNA. Hg^{2+} inhibits their activities, whereas PMSF and leupeptin do not. In contrast, the molecular mass of the

purified enzyme (270 kDa) is larger than that of the arginyl aminopeptidases of *Lactobacillus sakei* (180 kDa) (30), *Streptococcus gordonii* (70 kDa) (9) and *Debaryomyces hanseii* (101 kDa) (5), the optimal pH of the purified enzyme (6.5) is higher than that of *L. sakei* aminopeptidase (5.0) (30), and *S. gordonii* (5.6) (9), but lower than that of *D. hanseii* aminopeptidase (7.0) (5), and Zn^{2+} activates aminopeptidase from *C. granulosa* but inhibits those of both *L. sakei* (30) and *D. hanseii* (5).

The molecular mass of the purified native enzyme was estimated as 270 kDa by gel filtration. The subunit molecular mass was estimated as 86 kDa by SDS-PAGE, suggesting that the native enzyme consists of three identical polypeptides. Human placental aminopeptidase B (27) and *L. sakei* arginyl aminopeptidase (30) are also trimers, while *D. hanseii* arginyl peptidase is a dimer (5) and aminopeptidase B from human skeletal muscle (14), porcine liver (15) and porcine muscle (8) are monomeric.

The molecular mass and the four isoelectric points estimated by isoelectric focusing suggest the enzyme comprises four different trimers (ααα, ααβ, αββ, βββ) based on two kinds of subunits (α, β) with different charges. This type of protein pattern has not been reported in arginyl aminopeptidase, but has been reported in a purified metalloendopeptidase (6) and superoxide dismutases (1). Post-translational modifications such as methylation (29) and irreversible oxidation of sulfhydryl groups (21) might account for the differences in the charges.

The aminopeptidase purified in this study is an exo-type enzyme and is different from the endo-type enzymes which have previously been reported to be involved in calculus formation (23, 36, 37). However, results of one study suggest that exo-type aminopeptidase is involved in the process of mineralization of calculus (20). Further study is necessary to clarify the role of aminopeptidase in this regard.

In conclusion, aminopeptidase purified from culture supernatant of *C. granulosa* exhibits different characteristics from those enzymes purified from *Capnocytophaga* species in other studies, but similar characteristics to aminopeptidase B.

References

1. Amano A, Shizukuishi S, Tamagawa H, Iwakura K, Tsunasawa S, Tsunemitsu A. Characterization of superoxide dismutases purified from either anaerobically maintained or aerated *Bacteroides gingivalis*. J Bacteriol 1990: **172**: 1457–1463.
2. Andrews P. The gel-filtration behaviour of proteins related to their molecular weights over a wide range. Biochem J 1965: **96**: 595–606.
3. Barrett AJ, Rawlings ND, Woessner JF. Handbook of proteolytic enzymes. San Diego/Tokyo: Academic Press, 1998: 987–1550.
4. Böhlen P, Stein S, Dalrman W, Udenfriend S. Fluorometric assay of proteins related to their molecular weights over a wide range. Arch Biochem Biophys 1973: **155**: 213–220.
5. Bolumar T, Sanz Y, Aristoy M-C, Toldrá F. Purification and properties of an arginyl aminopeptidase from *Debaryomyces hanseii*. Int J Food Microbiol 2003: **86**: 141–151.
6. Butler PE, McKay MJ, Bond JS. Characterization of meprin, a membrane-bound metalloendopeptidase from mouse kidney. Biochem J 1987: **241**: 229–235.
7. Cadel S, Foulon T, Viron A, Balogh A, Midol-Monnet S, Noël N, Cohen P. Aminopeptidase B from the rat testis is a bifunctional enzyme structurally related to leukotriene-A_4 hydrolase. Proc Natl Acad Sci U S A 1997: **94**: 2963–2968.
8. Flores M, Aristoy MC, Toldrá F. HPLC purification and characterization of porcine muscle aminopeptidase B. Biochimie 1993: **75**: 861–867.
9. Goldstein JM, Nelson D, Kordula T, Mayo JA, Travis J. Extracellular arginine aminopeptidase from *Streptococcus gordonii* FSS2. Infect Immun 2002: **70**: 836–843.
10. Hartree EF. Determination of protein: a modification of the Lowry method that gives a linear photometric response. Anal Biochem 1972: **48**: 422–427.
11. Hirs CHW. Performic acid oxidation. Methods Enzymol 1967: **11**: 197–199.
12. Hopsu VK, Mäkinen KK, Glenner GG. A peptidase (aminopeptidase B) from cat and guinea pig liver selective for N-terminal arginine and lysine residues. I. Purification and substrate specificity. Acta Chem Scand 1966: **20**: 1225–1230.
13. Hopsu VK, Mäkinen KK, Glenner GG. A peptidase (aminopeptidase B) from cat and guinea pig liver selective for N-terminal arginine and lysine residues. II. Modifier characteristics and kinetic studies. Acta Chem Scand 1966: **20**: 1231–1239.
14. Ishiura S, Yamamoto T, Yamamoto M, Nojima M, Aoyagi T, Sugita H. Human skeletal muscle contains two major aminopeptidases: an anion-activated aminopeptidase B and an aminopeptidase M-like enzyme. J Biochem 1987: **102**: 1023–1031.
15. Kawata S, Takayama S, Ninomiya K, Makisumi S. Purification and some properties of porcine liver aminopeptidase B. J Biochem 1980: **88**: 1025–1032.
16. Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 1970: **227**: 680–685.
17. Laughon BE, Syed SA, Loesche WJ. API ZYM system for identification of *Bacteroides* spp., *Capnocytophaga* spp. & Spirochetes of oral origin. J Clin Microbiol 1982: **15**: 97–102.
18. Liu TY, Chang YH. Hydrolysis of proteins with p-toluenesulfonic acid. J Biol Chem 1971: **246**: 2842–2848.
19. Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. Protein measurement with the folin phenol reagent. J Biol Chem 1951: **193**: 265–275.
20. Malyshkina LT. Leucine aminopeptidase and aminotransferase activity in human dental calculus, dental deposit and saliva. Stomatologiia 1977: **56**: 5–9.
21. Mörikofer-Zwez S, von Wartburg JP, Aebï H. Heterogeneity of erythrocyte catalase: variability of the isoelectric point. Experientia 1970: **26**: 945–947.
22. Morishita M, Tokumoto K, Watanabe T, Iwamoto Y. Effect of a protease derived from the oral bacterium *Bacteroides loescheii* on the inhibition of calcium-phosphate precipitation by human parotid saliva. Arch Oral Biol 1986: **31**: 555–557.
23. Morita M, Watanabe T. Relation between the presence of supragingival calculus and protease activity in dental plaque. J Dent Res 1986: **65**: 703–705.
24. Morita M, Yamamoto T, Watanabe T. Effects of *Capnocytophaga gingivalis* to calcium-phosphate precipitation inhibitor of saliva *in vitro* [in Japanese]. J Dent Hlth 1991: **41**: 476–477.
25. Morita M, Yamamoto T, Watanabe T. Identification by biotinylated DNA probes of *Capnocytophaga* species isolated from supragingival calculus. J Dent Res 1991: **70**: 1048–1051.
26. Mort JS, Leduc M. A simple, economical method for staining gels for cathepsin B-like activity. Anal Biochem 1982: **119**: 148–152.
27. Nagata Y, Mizutani S, Nomura S, Kurauchi O, Kasugai M, Tomoda Y. Purification and properties of human placental aminopeptidase B. Enzyme 1991: **45**: 165–173.
28. Nakamura M, Slots J. Aminopeptidase activity of *Capnocytophaga*. J Periodontal Res 1982: **17**: 597–603.
29. Park KS, Frost BF, Shin S, Park IK, Kim S, Paik WK. Effect of enzymatic methylation of yeast iso-1-cytochrome *c* on its isoelectric point. Arch Biochem Biophys 1988: **267**: 195–204.
30. Sanz Y, Toldrá F. Purification and characterization of an arginine aminopeptidase from *Lactobacillus sakei*. Appl Environ Microbiol 2002: **68**: 1980–1987.
31. Shibata Y, Fujimura S, Nakamura T. Isolation and characterization of enzymes hydrolyzing chymotrypsin synthetic substrate (Enzyme I) and trypsin synthetic substrate (Enzyme II) from the envelope of *Capnocytophaga gingivalis*. Med Microbiol Immunol 1992: **181**: 107–115.
32. Simpson RJ, Neuberger MR, Liu TY. Complete amino acid analysis of proteins from a single hydrolysate. J Biol Chem 1976: **251**: 1936–1940.
33. Spratt DA, Greenman J, Schaffer AG. *Capnocytophaga gingivalis* aminopeptidase: a potential virulence factor. Microbiology 1995: **141**: 3087–3093.

34. Suido H, Eguchi T, Tanaka T, Nakamura M. Identification of periodontopathic bacteria based upon their peptidase activities. Adv Dent Res 1988: **2**: 304–309.
35. Suido H, Nakamura M, Mashimo PA, Zambon JJ, Genco RJ. Arylaminopeptidase activities of oral bacteria. J Dent Res 1986: **65**: 1335–1340.
36. Tokumoto K, Ohata N, Watanabe T, Iwamoto Y. The origin of the salivary protease correlated with calculus index [in Japanese]. Hiroshima Daigaku Shigaku Zasshi 1982: **14**: 11–14.
37. Watanabe T, Toda K, Morishita M, Iwamoto Y. Correlations between salivary protease and supragingival or subgingival calculus index. J Dent Res 1982: **61**: 1048–1051.
38. Yamamoto T, Kajiura S, Hirai Y, Watanabe T. *Capnocytophaga haemolytica* sp. nov. and *Capnocytophaga granulosa* sp. nov., from human dental plaque. Int J Syst Bacteriol 1994: **44**: 324–329.