

Carboxypeptidase Activity in Human Mycoplasmas

KEN-ICHIRO SHIBATA* AND TSUGUO WATANABE

Department of Oral Bacteriology, Nagasaki University School of Dentistry, 7-1 Sakamoto-machi, Nagasaki 852, Japan

Received 14 May 1986/Accepted 21 August 1986

***Mycoplasma salivarium* produced citrulline, ammonia, and ATP from *N*-benzoylglycyl-L-arginine. The activity was inhibited by EDTA and was therefore concluded to be due to an arginine-specific carboxypeptidase. The activity was also found to exist in *M. orale*, *M. buccale*, *M. faucium*, and *M. hominis*.**

Proteolytic activity has been demonstrated in *Mycoplasma salivarium* (10). The hydrolytic activity toward casein is weak and is inhibited by *o*-phenanthroline and EDTA. Therefore, the activity is presumed to be due to metallo-exopeptidases such as aminopeptidases or carboxypeptidases. Recently, aminopeptidase activity was found to exist in *M. salivarium* and was separated from caseinolytic activity by gel filtration (11).

The present study, designed to examine human mycoplasmas for carboxypeptidase activity, suggested that an arginine-specific carboxypeptidase existed in nonfermentative human mycoplasmas.

Most of the *Mycoplasma* strains tested were *Mycoplasma salivarium* ATCC 23064. Other mycoplasmas used for comparative purposes are listed in Table 1.

The media used were PLO broth (Difco Laboratories, Detroit, Mich.) with 1% (vol/vol) PLO serum fraction (SF medium) and the liquid medium of Hayflick (5) supplemented with 1% (wt/vol) glucose (G medium) or 1% (wt/vol) arginine hydrochloride (A medium).

M. fermentans and *M. pneumoniae* were grown in G medium, *M. salivarium* was grown in both A and SF media, and the other mycoplasmas were grown in A medium. Cells were harvested by centrifuging the cultures at $15,000 \times g$ for 15 min, washed three times, and suspended in a small volume of 0.25 M NaCl (cell suspensions [CS]). CS were kept at -70°C until used. When used, CS were thawed at 37°C and diluted appropriately with distilled water. Protein concentrations of CS were determined by the method of Lowry et al. (6).

Ninhydrin-positive substances (NPS) produced from *N*-benzoylglycyl-L-arginine (Bz-Gly-Arg), benzoylglycyl-L-lysine (Bz-Gly-Lys), and benzoylglycyl-L-histidine-L-leucine (Bz-Gly-His-Leu) (Protein Research Foundation, Osaka, Japan) were assayed by the colorimetric ninhydrin method described by Tsunasawa et al. (8) and expressed as the absorbance difference at 570 nm between the reaction mixtures with and without substrate (ΔA_{570}), measured by using a 1-cm cuvette. The buffer used was 0.1 M potassium phosphate buffer (pH 6.5).

Amino acid analysis of NPS was performed as follows. The reaction mixture, consisting of 2 ml of 5 mM potassium phosphate buffer, 2 ml of 5 mM Bz-Gly-Arg, and 1 ml of CS (0.28 mg of protein per ml) of *M. salivarium*, was incubated at 37°C for 40 min. Immediately after incubation and at intervals of 10 min, 1 ml of the mixture was withdrawn and added to 1 ml of 20% (wt/vol) trichloroacetic acid. The mixture was centrifuged at $1,800 \times g$ for 30 min, and 1 ml of

the supernatant was lyophilized. The lyophilized product was dissolved in 1 ml of 0.1 M citrate buffer (pH 2.2). The solution was diluted 1:20 with the same buffer and then applied to an automated Toyosoda model HLC 825-AA amino acid analyzer (Toyosoda Co., Ltd., Tokyo, Japan).

For the determination of ATP produced, the reaction mixtures, consisting of 0.15 ml of 0.1 M potassium phosphate buffer, 0.05 ml of 25 mM ADP, 0.2 ml of 25 mM Bz-Gly-Arg or 25 mM arginine, and 0.2 ml of CS (2.8 mg of protein per ml) of *M. salivarium*, were incubated at 37°C . Supernatants separated by centrifuging the reaction mixtures incubated for 0, 20, 40, and 80 min and those incubated for the same times without the substrate or CS were assayed for ATP content by using the ATP test (prepared in accordance with Bücher's description [3]) obtained from Boehringer GmbH, Mannheim, Federal Republic of Germany. Briefly, the ATP formed was detected by a decrease in the A_{340} due to the conversion of NADH to NAD by means of glyceraldehyde-3-phosphate dehydrogenase and glycerol-1-phosphate dehydrogenase.

NPS were shown to be produced rapidly from Bz-Gly-Arg by *M. salivarium* but not from Bz-Gly-Lys and Bz-Gly-His-Leu. NPS were considered to be arginine released from Bz-Gly-Arg by the action of a carboxypeptidase. To confirm this, we analyzed the supernatant of the reaction mixture with an amino acid analyzer. Unexpectedly, it was shown that the supernatant did not contain arginine but did contain citrulline (an intermediate product in the arginine dihydrolyase pathway) and ammonia. These products tended to increase in amounts parallel to the reaction time (Fig. 1). Thus, NPS were found to be citrulline and ammonia. In addition, ATP as well as arginine was shown to be produced from Bz-Gly-Arg (Fig. 2).

TABLE 1. NPS production from Bz-Gly-Arg by CS of human mycoplasmas

Mycoplasma tested ^a	Activity ^b
<i>M. pneumoniae</i> IID817 ^c	0.02
<i>M. fermentans</i> IID812 ^c	0.03
<i>M. faucium</i> IID996 ^c	4.12
<i>M. buccale</i> IID802 ^c	5.12
<i>M. salivarium</i> ATCC 23064	2.36
<i>M. orale</i> ATCC 15539	2.95
<i>M. hominis</i> IID801 ^c	0.72

^a *M. salivarium* and the other nonfermentative mycoplasmas were grown in A medium.

^b Expressed as the mean of three determinations of the $\Delta A_{570}/5$ min per mg of protein, measured by using a 1-cm cuvette.

^c Obtained from the Institute of Medical Science, University of Tokyo, Tokyo, Japan.

* Corresponding author.

On the basis of these results, it was presumed that no sooner than arginine had been produced from Bz-Gly-Arg by an arginine-releasing enzyme such as a carboxypeptidase, arginine was hydrolyzed by the enzymes in the arginine dihydrolase pathway. This was considered to be the reason that arginine was not detected in the reaction mixtures. To validate this speculation, we examined the effect of EDTA on NPS production, because this chelator is known to inhibit carboxypeptidases (4). NPS production was inhibited by EDTA (Fig. 3), suggesting that carboxypeptidase activity existed in *M. salivarium*.

The other human mycoplasmas were also examined for NPS production from Bz-Gly-Arg. Carboxypeptidase activity was found to exist in nonfermenters (*M. orale*, *M. buccale*, *M. faucium*, and *M. hominis*) but not in fermenters (*M. fermentans* and *M. pneumoniae*) (Table 1). Incidentally, mycoplasmas are divided into fermentative or nonfermenta-

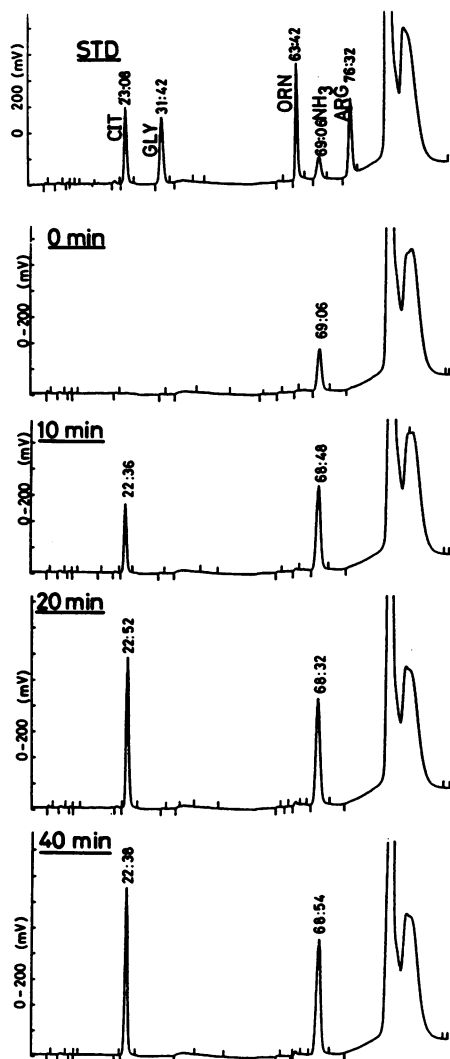


FIG. 1. Amino acid analyses of the reaction products obtained by incubating Bz-Gly-Arg with CS of *M. salivarium*. STD represents a chromatogram of the mixture of arginine (ARG), citrulline (CIT), glycine (GLY), and ornithine (ORN). 0, 10, 20, and 40 min represent chromatograms of the products obtained by incubating Bz-Gly-Arg with CS for 0, 10, 20, and 40 min, respectively. The numbers near the peaks represent the retention times. See the text for details.

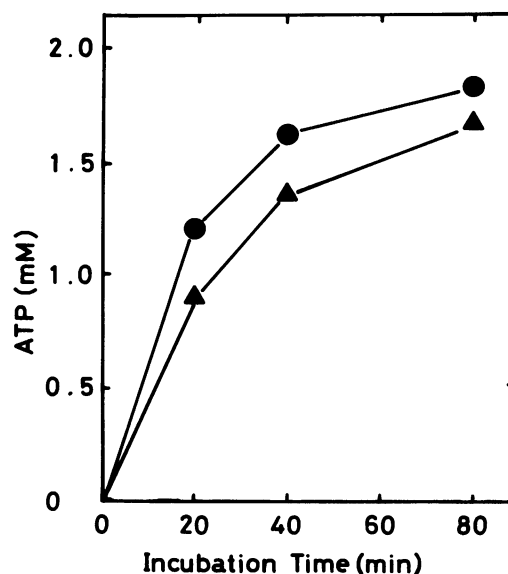


FIG. 2. ATP formation from ADP through the metabolism of arginine or Bz-Gly-Arg. CS diluted appropriately with distilled water were incubated with ADP and arginine or Bz-Gly-Arg at 37°C for 0, 20, 40, and 80 min. ATP contents were corrected with the contents obtained in control experiments. Symbols: ●, arginine; ▲, Bz-Gly-Arg.

tive species on the basis of acid production from carbohydrate. *M. fermentans* (2) is a fermentative species but is also a nonfermentative species (2, 7) and is known to possess the arginine dihydrolase pathway by which arginine is catabolized to ATP. Therefore, cells of the organism grown in A medium were also examined for carboxypeptidase activity. This activity ($\Delta A_{570}/5$ min per mg of protein, 0.02) was demonstrated to be almost equivalent to that (0.03, Table 1) in cells grown in G medium.

This is the first report of the existence of an arginine-specific carboxypeptidase in nonfermentative human

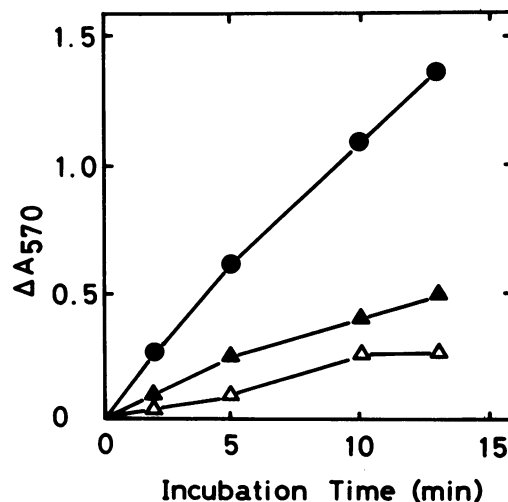


FIG. 3. Effect of EDTA on NPS production from Bz-Gly-Arg by CS of *M. salivarium*. CS (34 μ g of protein) were preincubated in the absence (●) or presence of 1 mM EDTA (▲) or 2 mM EDTA (△) at 37°C for 10 min, and then NPS production was assayed at regular intervals by the method described in the text.

mycoplasmas, although the activity is already known to exist in *Acholeplasma laidlawii*, a fermentative species (9). As indicated above, nonfermenters catabolize arginine and possess an arginine-specific aminopeptidase (1, 11). Judging from the arginine specificity of the carboxypeptidase and the aminopeptidase and from the well-known significance of the arginine dihydrolase pathway in nonfermentative mycoplasmas, these peptidases seem to be naturally occurring enzymes, perhaps functioning to supply arginine to the pathway. Further studies are in progress in our laboratory to purify and characterize the enzymes.

LITERATURE CITED

1. Ball, H. J., S. D. Neill, and L. R. Reid. 1982. Use of arginine aminopeptidase activity in characterization of arginine-utilizing mycoplasmas. *J. Clin. Microbiol.* **15**:28-34.
2. Barile, M. F., R. T. Schimke, and D. B. Riggs. 1966. Presence of the arginine dihydrolase pathway in *Mycoplasma*. *J. Bacteriol.* **91**:189-192.
3. Bücher, T. 1947. Über ein phosphatübertragendes Gärungsferment. *Biochim. Biophys. Acta* **1**:292-314.
4. Folk, J. E. 1970. Carboxypeptidase B (porcine pancreas). *Methods Enzymol.* **19**:504-508.
5. Hayflick, L. 1965. Tissue cultures and mycoplasmas. *Tex. Rep. Biol. Med.* **23**(Suppl. 1):285-303.
6. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193**:265-275.
7. Schimke, R. T., C. M. Berlin, E. W. Sweeny, and W. R. Carrol. 1966. The generation of energy by the arginine dihydrolase pathway in *Mycoplasma hominis* 07. *J. Biol. Chem.* **241**:2228-2236.
8. Tsunasawa, S., K. Narita, and K. Ogata. 1975. Purification and properties of acylamino acid-releasing enzyme from rat liver. *J. Biochem.* **77**:89-102.
9. Vinther, O., and F. T. Black. 1974. Aminopeptidase activity of *Ureaplasma urealyticum*. *Acta Pathol. Microbiol. Scand. Sect. B* **82**:917-918.
10. Watanabe, T. 1975. Proteolytic activity of *Mycoplasma salivarium* and *Mycoplasma orale* 1. *Med. Microbiol. Immunol.* **161**:127-132.
11. Watanabe, T., K. Shibata, and M. Totsuka. 1984. Aminopeptidase and caseinolytic activities of *Mycoplasma salivarium*. *Med. Microbiol. Immunol.* **172**:257-264.