PEPTIDASE ACTIVITY IN THE MEMBRANES OF MYCOPLASMA LAIDLAWII[1,2]

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SUMMARY: Membrane-bound peptidase activity was found in Mycoplasma laidlawii Similar activity was also found in the cytoplasm and to a small extent in the medium. Aminopeptidase and dipeptidase activities were present in the membranes as well as small amounts of carboxypeptidase activity.

In attempting to prepare peptide maps of plasma membranes of Mycoplasma laidlawii, we encountered an unexpected difficulty. When undenatured membranes were used, much of the protein was converted to free amino acids, with only small amounts of peptides. The source of the problem has been traced to the presence of exopeptidases within the membrane. The occurrence of exopeptidases in erythrocyte membranes has been known for a long time (1), but there is no record of their having created problems in peptide analysis. We were unable to find any account of peptidases in other Mycoplasmas, and few such membrane-associated enzymes have been described in bacteria. A particulate D-alanine carboxypeptidase has been found in Bacillus subtilis, and is thought to be concerned with the assembly of the bacterial cell wall material (2).

EXPERIMENTAL

Enzymes and peptides. Subtilisin was obtained from Nutritional Bio-

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[1] Edward and Freundt (3) propose that M. laidlawii be renamed Acholeplasma laidlawii and be assigned to a new family of the Mycoplasmatales in view of the unique properties of this organism.

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chemicals, Cleveland, Ohio. L-seryl-L-tyrosine amide HCl was obtained from Cyclo Chemical Co., Los Angeles, Calif.; N-acetyl-L-phenylalanyl-L-tryptophan and L-arginyl-L-alanine, acetate salt from Fox Chemical Co., Los Angeles, Calif.; L-leucine amide from BDH Ltd., Poole, England.

Standard peptide maps. Various membrane digests were prepared, and analyzed on standard sheets of Whatman No. 3MM chromatography paper (46 x 57 cm). The general procedure is similar to that described by Katz, Dreyer and Anfinsen (4). Descending chromatography was carried out first, in the shorter dimension, using n-butanol/acetic acid/water (4:1:5, by volume); running time was 14-17 hours. After the papers were dried, electrophoresis was carried out for $2\frac{1}{4}$ hours at 22°, at 1500 volts; the buffer used was pyridine/acetic acid/water (1:10:89, by volume), pH 3.6. Electrophoresis was performed in the long dimension using a Savant Model LT 48A system.

One-dimensional high voltage electrophoresis. Some membrane digests and peptide hydrolysates were examined by high-voltage electrophoresis at pH 1.6. Samples were applied to a 46 x 200 cm length of Whatman No. 3MM paper, and electrophoresis was carried out in a Gilson Model DW system at 43°. The buffer used was 7% formic acid, pH 1.6; running time was $1\frac{1}{2}$ hours at 7800 volts (5).

Staining of peptides. After thorough drying at 70°, the papers were stained with cadmium-ninhydrin (5) and photographed.

Preparation of membranes. *Mycoplasma laidlawii*, strain A, was grown in SP-YE medium as described by Pollock *et al.* (6) except that a 10-fold concentrate of the medium was frozen, thawed and filtered through diatomaceous earth to remove particulate matter. The medium was then centrifuged and diluted to normal strength before use. Membranes were prepared by an osmotic lysis procedure described previously (7).

Digestion of membranes. Membranes equivalent to 3.4 mg of protein were suspended in 0.5 ml of 0.1 M NH_4HCO_3 containing 0.2 mg of subtilisin, and allowed to digest overnight at 37°. No divalent cations were added. Thymol

was added to retard bacterial growth. They were then lyophilized and dissolved in 0.5 ml of 7% formic acid for application to the chromatography paper. Other digestion procedures and controls are dealt with in the results section.

Amino acid analyses. Samples of membranes and cytoplasm which had been digested overnight at 37° with subtilisin were analyzed for free amino acids by ion-exchange chromatography on a Beckman Model 121 Amino Acid Analyzer.

Digestion of peptides by membranes. Each digestion mixture contained 3 mg of membrane protein and 1 ml of 20 mM peptide solution in 0.1 M NH_4HCO_3 buffer containing 10^{-3} M $CaCl_2$ and 10^{-3} M $MgCl_2$. Digestion was allowed to proceed overnight at 37° in the presence of thymol. Mixtures were then lyophilized and dissolved in 1 ml of 7% formic acid. Samples equivalent to 50 nmoles of peptide were then analyzed by high-voltage electrophoresis at pH 1.6

RESULTS

A typical peptide map of subtilisin-digested membranes is shown in Fig. 1a. It is clear that much of the protein has been degraded to free amino acids. This batch of enzyme produced no free amino acids when tested against performic acid-oxidized ribonuclease; the same result was obtained with a different batch of subtilisin. Bacterial contamination of the digests was found to be negligible by microscopic examination and plating techniques. It thus seemed likely that there was peptidase activity in the membrane preparation, resulting in degradation of the subtilisin-produced peptides to free amino acids.

Samples of membranes (3.4 mg protein) were then analyzed without subtilisin digestion. One sample was stored at 0° in 0.1 M NH_4HCO_3, while a second was incubated overnight at 37° using the same conditions as were used for the enzymatic digestion, except for the absence of subtilisin. They were then lyophilized and examined by the standard peptide-mapping procedure. It will be seen from Fig. 1b that there was no significant contami-

nation of the membranes by free amino acids; overnight incubation (Fig. 1c) produced small amounts of free amino acids, but no peptides. This suggested strongly that the membranes contained exopeptidase activity, but little or no endopeptidase activity. Digestion of the membrane proteins by subtilisin produces a complex mixture of peptides which are rapidly degraded by the membrane exopeptidases.

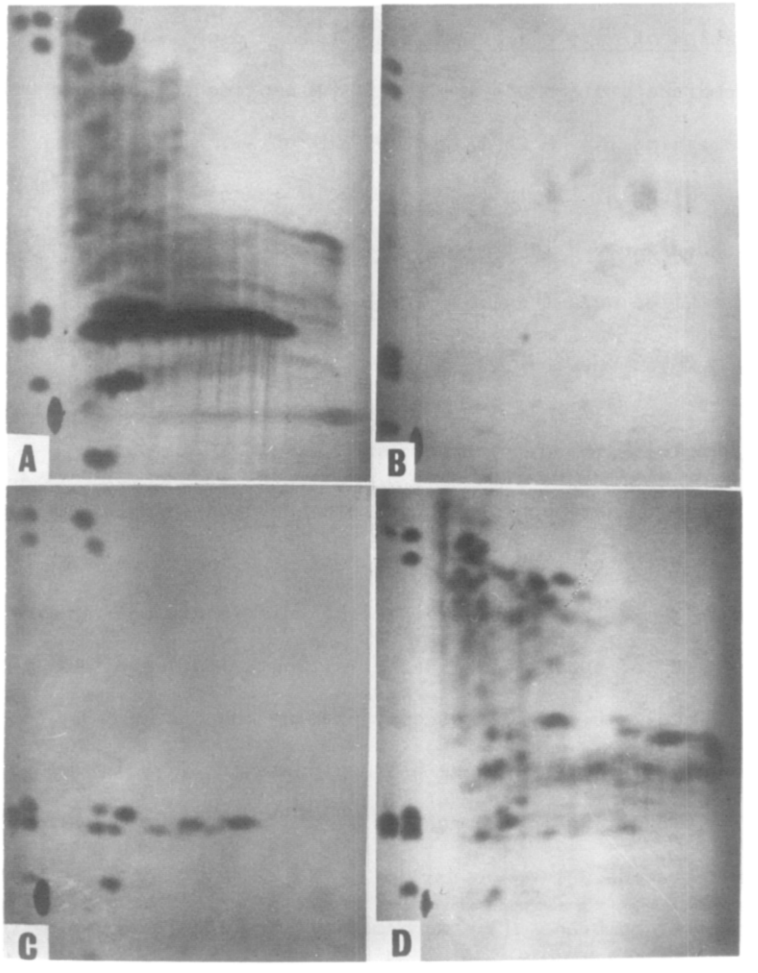

Fig. 1. Peptide maps of M. laidlawii membranes. Origin is at bottom left; chromatography in horizontal dimension; electrophoresis in vertical dimension, cathode at top; amino acid standards were also electrophoresed, and are visible at the left of each sample. A, 3.4 mg of undenatured membrane protein, digested with subtilisin. B, 3.4 mg of membrane protein stored undigested at 0° C. C, 3.4 mg of membrane proteins incubated overnight at 37° C in 0.1 M NH_4HCO_3. D, 1.8 mg of membrane proteins denatured by boiling (96° C) for ten minutes in presence of 10^{-3} M EDTA, then digested by subtilisin.

Various procedures were tested as a means of destroying the peptidase activity so that satisfactory peptide maps could be obtained. Membranes were treated in various ways, digested with subtilisin, and then analyzed by high-voltage electrophoresis at pH 1.6. Acetone precipitation, perchloric acid extraction, and treatment with 10^{-3} M EDTA did not fully inactivate the peptidase, as judged by the presence of free amino acids in the digest. Boiling was found to be effective, however, especially in the presence of 10^{-3} M EDTA. Fig. 1d shows a peptide map of membranes which were heated at 96° for 10 minutes (boiled) in the presence of 10^{-3} M EDTA, and then digested with subtilisin. Only small amounts of free amino acids are present.

The dialysed cytoplasm and growth medium were also tested for peptidase activity by preparing peptide maps of subtilisin-digested material. There was considerable activity in the cytoplasm, but much less in the medium as judged by the relative amounts of free amino acids and peptides.

The nature of the peptidase activity was investigated briefly using some synthetic peptides that we had available. Table 1 lists the approximate degree of splitting of the various substrates after overnight incubation at 37°. From the limited range of substrates tested, it appears likely that the major activities present are aminopeptidase and dipeptidase, with much weaker carboxypeptidase.

TABLE 1. Hydrolysis of peptides[1] by Mycoplasma membranes.

Substrate	Products[2]	Degree of Hydrolysis	Activity
Arg.Ala	Arg, Ala	>90%	Dipeptidase
Ser.Tyr.NH_2	Ser, Tyr, Tyr.NH_2(?)	20-25%	Aminopeptidase
Leu.NH_2	Leu	80-90%	Aminopeptidase
Ac.Phe.Trp	Trp	<10%	Carboxypeptidase

[1] To 1 ml of 20 mM substrate in 0.1 M NH_4HCO was added 3 mg membrane protein and 1 µmole each $CaCl_2$ and $MgCl_2$ followed by incubation overnight at 37°.

[2] Only ninhydrin positive products are listed.

Quantitation of the free amino acids produced by subtilisin and exopeptidase acting on membranes and cytoplasmic protein was also revealing. With one preparation the digests were remarkably high in valine and isoleucine, and remarkably low in glycine and alanine compared with their content in whole membranes (Table 2). There was also a roughly ten-fold preference for isoleucine versus leucine. A second preparation of Mycoplasma did not show such a high selectivity.

TABLE 2. Release of free amino acids from Mycoplasma membranes and cytoplasmic proteins.[1]

Amino Acid	Membranes			Cytoplasmic Proteins		
	Acid (nmoles)	Sample 1 (% of acid)	Sample 2 (% of acid)	Acid (nmoles)	Sample 1 (% of acid)	Sample 2 (% of acid)
Asp[2]	480	3.1	8.1	720	5.4	3.0
Thr	260	4.2	28	450	7.8	38
Ser[2]	270	20	29	325	35	46
Glu[2]	330	13	17	715	8.9	2.9
Pro	165	0.0	0.0	275	0.0	3.0
Cys	0	0	0	0	0	0
Gly	350	0.7	N.D.	680	0.9	25
Ala	385	0.5	18	470	0.7	58
Val	280	65	68	485	58	40
Met	17	6.2	26	trace	-	-
Ile	260	74	44	365	75	43
Leu	420	7.5	21	580	14	39
Tyr	180	11	51	210	6.4	50
Phe	220	11	61	238	20	50

[1] Membranes or dialysed cytoplasm were hydrolysed by acid (6N HCL, 22 hours, 110°) or by digestion with subtilisin and intrinsic peptidases (for conditions, see text). Amounts of sample applied to the amino acid analyzer were equivalent to 0.75 mg of protein as determined by the Lowry method. Two preparations of Mycoplasma were used which did not differ significantly in their acid hydrolysates. Only the neutral and acidic amino acids are listed because small peptides chromatographed with the basic amino acids and made interpretation impossible. Values listed in the table are the percentages of the various amino acids released by enzyme digestion, relative to the amount released by acid (e.g., with membrane sample 1, 192 nmoles of Ile were released = 74% of 260 nmoles).

[2] Asn and Gln are not distinguished from Asp and Glu by acid hydrolysis, and are not distinguished from Ser after enzyme digestion.

DISCUSSION

We have shown clearly that there is peptidase activity associated with the membrane of Mycoplasma laidlawii. Whether this activity is an intrinsic part

of the membrane is a question that we have not attempted to answer directly. Since there is considerable activity in the cytoplasm, it might be thought that the activity associated with the membrane is merely adsorbed passively during the isolating procedure. This seems unlikely, for we have shown previously that cytoplasmic proteins are not exchangeable with the membrane proteins, or adsorbed by re-exposure to membranes (7). It also seems unlikely that the enzyme is being synthesized within the cell for export to the external medium, since relatively little activity was found in the latter.

Tests of activity were limited to only a few substrates, but it appears that the activity is likely to be predominantly aminopeptidase rather than carboxypeptidase. This is borne out by the data in Table 2. Due to its specificity, subtilisin would most often expose leucine, methionine, tyrosine, and phenylalanine as carboxy-terminal amino acids. In contrast, valine and isoleucine residues would not be cleaved on the carboxyl side and therefore would appear more often near the amino-terminal end. It is unlikely that the preference for valine and isoleucine is due to any unusual structural feature of the membrane proteins, for the same basic pattern of release was observed with cytoplasmic proteins. The pattern of release with the second preparation of Mycoplasma suggests that there was also a large amount of carboxypeptidase activity present.

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