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THE EFFECT OF PROTEASES ON MEMBRANE ATPase FROM TWO LACTIC ACID BACTERIA

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SUMMARY

Cell membranes from *Lactobacillus fermenti* were prepared by a combined protease–lysozyme treatment using trypsin, chymotrypsin or subtilisin. With either protease during lysis, a large part of the ATPase activity, approx. 60–80 % of the total, was recovered in the soluble cell fractions. However, the specific activity of ATPase was much higher in the membranes than it was in the soluble cell fractions. When Mg^{2+} was omitted from the medium employed during lysis of the cells most of the ATPase was recovered in the soluble cell fractions. The activity of the corresponding membrane preparations was only a few per cent of the total.

All three proteases were equally efficient in rendering the cells susceptible to lysozyme. However, they had a different effect on the ATPase in the isolated membranes and also on that present in other cell fractions. Subtilisin appeared to exert a more powerful proteolytic action than did trypsin, but the trypsin treatment resulted in membranes and soluble cell fractions with considerably lower ATPase activity than did treatment with subtilisin (or chymotrypsin).

Once prepared in the presence of adequate amounts of Mg^{2+} only about half of the membrane ATPase could be solubilized by extensive washing with water alone or with 50 mM Tris–HCl. However, it could be completely released from the membrane in the presence of 1 mM EDTA.

Cell membranes from *Streptococcus faecalis* were prepared by treatment with lysozyme. The ATPase activity in such preparations considerably decreased in the presence of trypsin and somewhat less so in the presence of subtilisin. ATP had a protective effect towards both proteases. This effect was more pronounced with trypsin than it was with subtilisin.

ATP decreased the extent of lysis of *L. fermenti* in the presence of lysozyme and protease. Membranes prepared from such lysates had considerably higher ATPase activity than those from suspensions lysed without ATP.

INTRODUCTION

Unlike other Gram-positive bacteria that have been investigated, *Lactobacillus fermenti* is not directly susceptible to the action of lysozyme[1]. However, treatment with trypsin was found to render the cells lysozyme-sensitive[2]. The ATPase in mem-

branes isolated by such combined trypsin–lysozyme treatment had lower activity than that in earlier preparations of particulate cell fractions obtained by mechanical disruption of cells[1]. This gave the incentive to investigate the effect of other proteases. The present paper reports the results of these studies. It also describes comparative experiments with cell membranes from *Streptococcus faecalis* prepared by treatment with lysozyme only.

MATERIALS AND METHODS

Reagents

The following were preparations from Sigma, St. Louis, Mo., U.S.A.: Egg white lysozyme, trypsin, α-chymotrypsin (Type II), subtilisin (bacterial subtilopeptidase-A, Type VII), deoxyribonuclease, adenosine 5′-triphosphate disodium salt (ATP), adenosine 5′-diphosphate sodium salt (ADP) and adenosine 5′-monophosphate sodium salt (AMP). Sodium pyrophosphate, reagent grade, and EDTA were purchased from Merck (Darmstadt, Germany). All other chemicals were also of reagent grade. Redistilled water was used throughout the investigation.

Organisms and growth media

The maintenance, storage and cultivation of *L. fermenti* 36 (ATCC 9338) were carried out as previously described[3]. The content of thiamine in the culture medium was 2 mg/l. *S. faecalis* (ATCC 9790) was grown in a medium of similar composition, but with 1 mg thiamine/l. The cells of both organisms were usually grown for 12–16 h before harvesting employing two-step cultivation; *L. fermenti* at 37°; *S. faecalis* at 30°.

Preparation and lysis of washed cell suspensions

The cells were harvested by centrifugation. They were washed once with 0.9 % NaCl, and twice with redistilled water. They were re-suspended in Tris–HCl buffer (pH 7.5), 50 or 100 mM, with 10 mM $MgCl_2$ or without Mg^{2+}, as indicated. The initial density of the cell suspensions was usually adjusted to 4–6 % transmittance in a Coleman spectrophotometer, Model 14. The cell suspensions of *L. fermenti* were incubated with the respective proteases (200 μg/ml) and lysozyme (400 μg/ml) at 45°. In many experiments, all three proteases were tested in simultaneous incubations using aliquots of the same cell suspension. The cells of *S. faecalis* were incubated with lysozyme only (400 μg/ml) at 37°.

The extent of lysis was measured by turbidimetric determinations. In certain experiments this was followed by microscopic examination. All determinations were carried out in duplicate or triplicate. Each experiment was repeated at least once.

Preparation of membrane-like fragments

The transmittance of the suspensions usually increased from about 5 to about 70–90 % after 2–3 h. They were then centrifuged at 38000 × *g* for 15 min. The sediment was washed with a buffer consisting of Tris–HCl (50 or 100 mM, pH 7.5) and $MgCl_2$ (10–50 mM). Washing was continued untill the protein content of the wash medium was less than 20 μg/ml. This was estimated from the ratio of the absorbances at 280 and 260 mμ[4]. Usually 4–7 successive washings were required. A trace of deoxy-

ribonuclease (1–2 μg/ml) was added during the first washing in order to decrease the viscosity of the suspension. When necessary to remove any whole cells, the suspension was centrifuged at low speed (2000–3000 $\times$ *g*) before the last washing. The supernatant was then centrifuged again at 38000 $\times$ *g* to sediment the membrane material. In some experiments Mg^{2+} was omitted from the suspension to be lysed and from the subsequent washings of the membrane fraction. This was done in order to release the ATPase from the membrane. The final sediment was re-suspended in 5–7 ml of buffer (50 or 100 mM Tris–HCl, pH 7.5) and stored at 4° until analyzed for protein content and ATPase. Protein was determined according to LOWRY *et al.*[5], crystalline bovine serum albumin being used as standard. The supernatants from the lysed suspension and from all washings were recovered. They were analyzed for protein content and for ATPase. If necessary, the analysis was done after freeze-drying and de-salting on Sephadex G-25 columns.

ATPase assays

The reaction mixture had a total volume of 2 ml. It contained: 100 mM Tris–HCl (pH 7.5), 5 mM ATP (disodium salt), 25 mM $MgCl_2$, and 50–125 μg/ml membrane protein. The mixture was prepared in centrifuge tubes cooled in ice. Incubation was carried out at 37° during 10 or 20 min as indicated. The reaction was stopped with 1 ml ice-cold 1 M $HClO_4$, and the tubes were immediately transferred to an ice bath. The protein precipitate was centrifuged off. P_i was determined in the supernatant according to the method of Berenblum and Chain as modified by MARTIN AND DOTY[6] (revised by LINDBERG AND ERNSTER[7]). The values obtained were corrected for the spontaneous decomposition of the substrate and for the endogenous phosphate content of the respective preparations. All ATPase determinations were carried out in duplicate. Each membrane preparation was tested at least twice.

RESULTS

Lysis of L. fermenti with lysozyme and proteases. ATPase in the corresponding membranes

Fig. 1 shows the course of lysis of washed *L. fermenti* cells using trypsin, chymotrypsin or subtilisin. It can be seen that all three proteases are equally efficient in rendering the cells sensitive to lysozyme. The action of trypsin during the first hour is possibly more rapid than the action of the other two proteases. However, considerable differences are found in the ATPase activity of the membrane preparations. These are illustrated in Table I. There is much less ATPase activity in the membranes prepared with trypsin than in those prepared with chymotrypsin or subtilisin. Trypsin is highly efficient with respect to cell lysis (*cf.* Fig. 1). The lower activity of the preparations with trypsin cannot therefore be due to differences between the proteolytic power of the three proteases. Rather, it indicates that trypsin may be more efficient than the other two proteases with respect to either release or destruction of the membrane ATPase.

Distribution of protein and ATPase in cell fractions from L. fermenti

In order to test the first-mentioned possibility the distribution of total proteins and ATPase was studied in cell fractions prepared with either trypsin or subtilisin,

in the presence or in the absence of Mg^{2+}. The results indicated that subtilisin had a more extensive degrading effect than trypsin on the proteins of all cell fractions.

When prepared in the presence of Mg^{2+} and either trypsin or subtilisin, the membranes exhibited a higher specific activity of ATPase than the soluble cell fractions. In the preparations with subtilisin, the specific activity of ATPase in all fractions was much higher than in the corresponding preparations with trypsin. In the absence of Mg^{2+} the ATPase activity in both cases was almost completely solubilized.

The results of balance studies on the distribution of total proteins and total ATPase indicated that when either protease was used only a minor part of the total ATPase activity remained associated with the membrane fraction. This part was about 25 % in the preparations with trypsin and about 15 % in the preparations with subtilisin. With subtilisin a major part (75 %) of the total activity was recovered directly in the soluble cell content. In contrast, with trypsin the activity was successively removed from the membrane upon consecutive washings, giving extracts with increasing contents of ATPase.

In some experiments the content of Mg^{2+} during the successive washings of the membrane was increased to 50 mM from the 10 mM usually employed. The

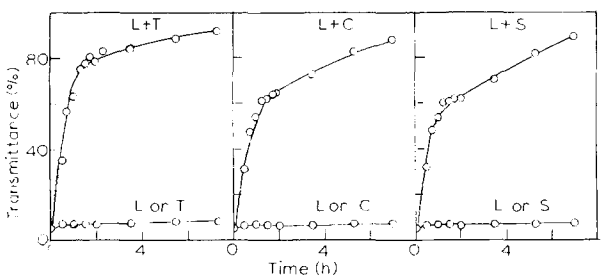

Fig. 1. Lysis of washed cell suspensions of *L. fermenti* in the presence of lysozyme and protease. Protease, 200 μg/ml; lysozyme (L), 400 μg/ml; $1 \cdot 10^9$–$1 \cdot 10^{10}$ cells/ml. Incubation at 45° in 100 mM Tris–HCl (pH 7.5) together with 10 mM $MgCl_2$. T, trypsin; C, chymotrypsin; S, subtilisin.

TABLE I

THE INFLUENCE OF PROTEASES ON THE ACTIVITY OF ATPASE IN CELL MEMBRANES PREPARED FROM *L. fermenti* BY COMBINED PROTEASE–LYSOZYME TREATMENT

Lysis at 45° for 2.5–3.5 h in 100 mM Tris–HCl (pH 7.5) together with 10 mM $MgCl_2$. Protease, 200 μg/ml; lysozyme, 400 μg/ml. Preparation of membranes in the same buffer without enzymes. ATPase assay as described in the text.

Protease used during incubation of the washed cells with lysozyme	*Activity of ATPase in the membranes subsequently isolated (μmoles P_i/g protein per min)*			
	Expt. 1	*Expt. 2*	*Expt. 3**	*Expt. 4**
Trypsin	6.9	27.5	7.3	12.0
Chymotrypsin	21.3	56.8	61.0	---
Subtilisin	28.8	76.8	67.0	75.0

* 50 mM Tris–HCl.

proportion of ATPase associated with the membrane then increased to some extent. However, substantial amounts were always recovered in the soluble fractions.

It was concluded from these results that the membrane ATPase is released by both subtilisin and trypsin. In order to explain the lower activity of ATPase in membranes and other cell fractions prepared with trypsin, studies were undertaken on a possible destruction of ATPase by these proteolytic enzymes.

Destruction of membrane ATPase by trypsin and subtilisin. Protection by ATP

This possibility was tested by comparative experiments with membranes from *S. faecalis*, as such membranes can be prepared without using a protease[8–10]. The results are summarized in Figs. 2A–2C. Fig. 2A shows ATPase activity when the membranes were pre-incubated with either trypsin or subtilisin for 2 min before the addition of ATP. 10–100 μg of either protease per 100 μg of membrane protein destroyed large amounts of ATPase. A considerable protective effect of ATP, when it was added 2 min before the protease, was observed after incubation with trypsin.

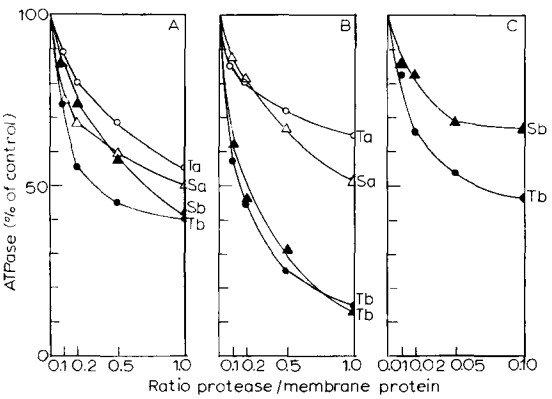

Fig. 2. The destructive effect of trypsin and subtilisin on membrane ATPase from *S. faecalis*, showing the protective effect of ATP. ATPase activity expressed as % of control without protease. The control values varied between 0.35 and 0.45 μmole P_i/mg protein per min using different preparations. Reaction mixture, 2 ml, containing (in μmoles): Tris–HCl, 200; ATP, 5; $MgCl_2$, 2.5; membrane protein, 100 μg; protease, 0–100 μg. The reaction was initiated by the addition of Mg^{2+}. Incubation for 20 min at 37°. T_b, S_b = trypsin or subtilisin added before ATP. T_a, S_a designate the same proteases added after ATP. Pre-incubation with protease or ATP at 20°: A, 2 min; B and C, 10 min.

This effect was less pronounced with subtilisin. The destructive effect of the two proteases, when added before ATP, appears even more distinctly in Fig. 2B. It shows a corresponding experiment involving incubation for 10 min instead of 2 min. It also shows the protective effect of ATP, added before protease.

With the highest amounts of protease (100 μg/100 μg membrane protein), the protective effect of ATP was more pronounced with trypsin than with subtilisin. However, at the lower concentrations of protease the two effects were comparable. Fig. 2C demonstrates, finally, that the two proteases act on the membrane ATPase even at concentrations as low as 1–2 μg/100 μg membrane protein. It also shows that trypsin is more detrimental to the ATPase than is subtilisin.

ATP obviously protects the membrane ATPase against proteolytic enzymes.

For this reason an attempt was made to protect the *L. fermenti* membranes by adding ATP during lysis with lysozyme and protease. As seen in Fig. 3, ATP (10 mg/ml) considerably decreased the rate of lysis. Lower amounts were without effect. Table II shows the P_i-releasing activity of membranes obtained from such lysates. The ATPase seemed to be present in much higher amounts than in preparations obtained by lysis without ATP. No experiments were carried out to elucidate whether this difference depends on the protection by ATP towards proteolytic destruction or towards release of the ATPase.

Other properties of the membranes from L. fermenti

The effect of Ca^{2+} and Mg^{2+} on the membrane ATPase from *L. fermenti* is

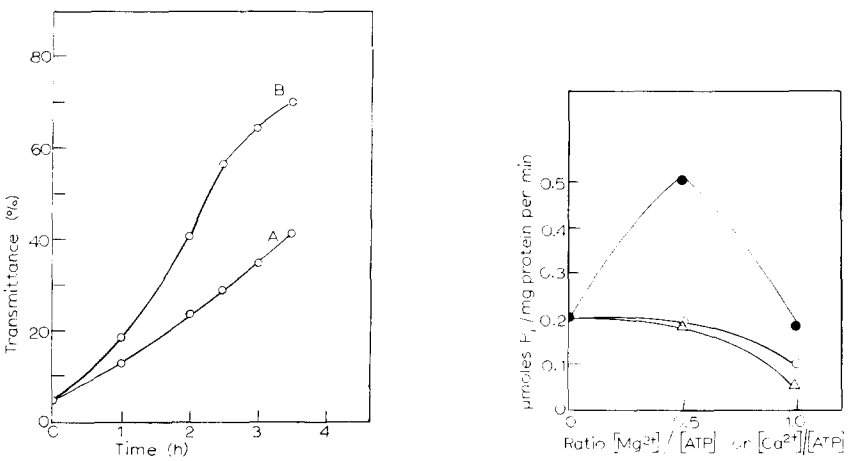

Fig. 3. Lysis of *L. fermenti* with subtilisin (200 µg/ml) and lysozyme (400 µg/ml) in the presence of ATP (10 mg/ml, Curve A), and in the absence of ATP (Curve B). Other conditions as for Fig. 1. Lower amounts of ATP (0.1–1.0 mg/ml) were without effect.

Fig. 4. The effect of Ca^{2+} on the activity of ATPase in membranes from *L. fermenti*. Reaction mixture as in Fig. 2, but without protease, with variable Ca^{2+} or/and Mg^{2+} and with 125 µg membrane protein. Initiated by the addition of ATP. ●—●, Mg^{2+}; ○—○, Ca^{2+}; △—△, Mg^{2+} + Ca^{2+}.

TABLE II

ATPASE ACTIVITY OF MEMBRANES FROM *L. fermenti* PREPARED BY LYSIS WITH LYSOZYME AND SUBTILISIN

Medium during lysis 50 mM Tris–HCl (pH 7.5) and 10 mM Mg^{2+}, in the presence of 10 mg ATP/ml and in its absence. The concentration of Mg^{2+} during preparation of membranes was 50 mM. Other conditions as for Fig. 1.

Preparation	*P_i liberated in the presence of ATP (µmoles P_i/g protein per min)*
Membrane	56
Membrane after washing with water (4 times)	28
Membrane (+ ATP)	251
Membrane (+ ATP) after washing with water (4 times)	152

shown in Fig. 4. Mg^{2+} was required for the activity. The optimal Mg^{2+}: ATP ratio was approx. 1:2. Ca^{2+}, at equimolar concentrations, inhibited the Mg^{2+}-activated ATPase. All this is in agreement with results of earlier studies on the ATPase activity of cell fragments obtained by mechanical disruption of *L. fermenti*[3]. The activating effect of Ca^{2+} alone amounted in such particles to approx. 40% of the Mg^{2+} effect[3]. This effect is probably 'masked' by the residual Mg^{2+} present in the lytic membrane preparations described here (*cf.* Fig. 4).

The substrate specificity of the ATPase in various fractions prepared in the presence of subtilisin was studied using ATP, ADP, AMP and PP_i. The activity of the washed membranes was mainly due to true ATPase. However, the activity of the soluble cell contents and of the consecutive washes was partly or totally due to inorganic pyrophosphatase. The activities of all fractions towards ADP and AMP were very small. The corresponding cell fractions prepared in the presence of trypsin released P_i in the presence of ATP only. ADP, AMP and PP_i were inactive as substrates.

When the final membrane preparations were subjected to extensive washing with water or 25 mM Tris–HCl (pH 7.5) about half of their ATPase activity was removed. In the presence of EDTA (1 mM), however, all the ATPase activity was detached from the membrane. These results are summarized in Table III (see also Table II).

TABLE III

THE RELEASE OF PROTEIN AND ATPASE FROM MEMBRANES OF *L. fermenti* PREPARED BY COMBINED TREATMENT WITH LYSOZYME (400 μg/ml) AND SUBTILISIN (200 μg/ml)

Medium during lysis: 0.1 M Tris–HCl (pH 7.5) containing 10 mM Mg^{2+}. Medium during preparation of the membranes: 50 mM Tris–HCl (pH 7.5) containing 50 mM Mg^{2+}; 6–7 successive washings. 2 ml of the final membrane preparations were re-suspended and washed twice as indicated, then twice more with water. There was no detectable protein or ATPase in the two last washes.

Membrane preparation No.	*Re-suspended and washed with*	*Associated with the membrane before and after washing*			
		Protein (mg)		*ATPase (units)***	
		before	*after*	*before*	*after*
1	Water	3.38	2.34	222	89
2*	Water	3.48	2.20	940	386
3	Tris–HCl (25 mM, pH 7.5)	3.30	1.90	315	142
4	Tris–HCl (25 mM, pH 7.5) EDTA (1 mM)	3.08	1.22	216	0

* From cells lysed in the presence of ATP (10 mg/ml).

** 1 unit of ATPase is defined as that amount of the enzyme which in the presence of ATP releases 1 μmole P_i/g protein per min.

DISCUSSION

The membrane-like particles prepared in the present study and the previously investigated particles from mechanically disrupted cells[3] are similar in their specificities towards various phosphorylated substrates. There are also great similarities

with respect to the distribution of the ATPase between the soluble and particulate cell fractions. In both cases a large part of the total activity was recovered in the soluble cell fraction, about 70–80 %. It is hard to decide whether this represents a true distribution of the ATPase activity in the cell. The previously studied particles from mechanically disrupted cells were prepared without added Mg^{2+}. However, they were not subjected to any extensive washing procedures likely to remove the Mg^{2+} already bound to the membranes. The ATPase present would be in the fragments composed of tightly interconnected cell wall and cell membrane. Any extensive release of the ATPase from such fragments does not seem probable (see *e.g.* Table III, this paper).

On the other hand, it may be the protease that is responsible for the release of ATPase from membrane fragments prepared by the lytic procedure described in this paper. This would be analogous to the reported behaviour of ATPase in the mitochondrial membrane when subjected to the action of trypsin (for review see ref. 11).

According to ABRAMS AND BARON[12] the ATPase in *S. faecalis* is attached to the membrane through Mg^{2+}. However, it has been observed in certain other microorganisms that ATPase may also occur in the space between the cell wall and the cell membrane. Such periplasmic ATPase would, of course, be released upon enzymic removal or mechanical disruption of the cell wall. Nevertheless, a part of it could conceivably remain attached to the membrane. The proportion would depend not only on the Mg^{2+} concentration but also on the availability of Mg^{2+}-complexing sites.

SALTON *et al.*[13] and MUÑOZ *et al.*[14] have shown that ATPase is a well defined component of the membrane itself in *Micrococcus lysodeikticus*, whereas polynucleotide phosphorylase (using ADP as substrate) is not. These authors demonstrated that ATPase could ultimately be released from the membrane of *M. lysodeikticus* by removal of divalent cations, low ionic strength during washing, *etc.* However, they found that this release is by no means a simple 'all or none' effect as has been suggested for *S. faecalis*[12,15–16].

The present results with *L. fermenti* also indicate that a major part of the membrane ATPase is released in the absence of Mg^{2+}. There is always a small residual activity however which, in the case of membranes prepared with lysozyme and subtilisin, cannot be released at all, and which is released very slowly from membranes prepared with lysozyme and trypsin. This view is also supported by the necessity to use EDTA in order to remove completely ATPase from membranes, prepared in the presence of Mg^{2+} (Table III). All these results indicate that there may in *L. fermenti* exist two different species of ATPase, one of them more tightly associated with the membrane than the other.

Cell fractions obtained from *L. fermenti* by controlled lysis with lysozyme and a protease had considerably lower ATPase activity than either the particulate or the soluble fractions obtained in an earlier study by disruption of frozen cells[1]. Comparative studies with membranes from *S. faecalis* (Fig. 2), indicate that this could be due to the destructive action of the proteases employed to render the cells of *L. fermenti* sensitive to lysozyme.

The difference between trypsin and subtilisin with respect to the ATPase activities in various cell fractions could be explained in more than one way. In the

case of *L. fermenti* one could suggest that trypsin releases ATPase from the membrane in a more efficient way than does subtilisin. The solubilized ATPase is then inactivated by the 45° incubation necessary to lyse the cell wall. However, the results of balance studies indicate that trypsin releases the ATPase in a less abrupt way than subtilisin. This would contradict the above explanation.

A corresponding explanation does not seem plausible for *S. faecalis* (Fig. 2). Preparation of membranes from this organism is carried out at 37°, and so also is the ATPase assay. According to ABRAMS[15] the ATPase from *S. faecalis* is easily solubilized by removal of divalent cations. The solubilized enzyme is remarkably stable under the assay conditions but it is inactivated by cold.

Other possible explanations of the differences between trypsin and subtilisin could be a different degree of activation or a different degree of destruction of the ATPase. It appears from Fig. 2, however, that no activation by either of the two proteases takes place with the ATPase of *S. faecalis*. A corresponding test with *L. fermenti* is not possible, at present, because 'reference' membranes cannot be prepared without a protease.

Thus, it would seem that a more specific destruction of the ATPase by trypsin than by subtilisin is the most plausible explanation of the observed differences.

It is interesting to recollect in this connection the suggestion of SOMOGYI[17] that trypsin is more destructive than other proteases in attacking the active centre of a (Mg^{2+}-Na^{+}-K^{+})-ATPase from rat brain.

According to N.S. GELMAN (personal communication) the ATPase in membranes from *M. lysodeikticus* is neither destroyed nor removed by subtilisin. According to MUÑOZ *et al.*[14,18,19] a Ca^{2+}-dependent ATPase, when associated with membranes of *M. lysodeikticus*, was only able to act on ATP if activated by trypsin. The ATPase had to be protected, however, by a prior addition of ATP. These findings would seem at variance with the observations concerning the ATPase of *L. fermenti* and *S. faecalis*. The ATPase activity in membranes from *S. faecalis* decreases considerably in the presence of trypsin and somewhat less so in the presence of subtilisin (Figs. 2A–2C). ATP exerts a protective effect towards both proteases but no increase of the ATPase activity above the control is observed in the presence of ATP.

Thus there would seem to be fundamental differences between the ATPase from *L. fermenti* or S. *faecalis* on the one hand and that from *M. lysodeikticus* on the other. The first type of ATPase is dependent on Mg^{2+} for activity and it is destroyed by trypsin (Figs. 2 and 4). The second type is dependent on Ca^{2+} and is activated by trypsin[14,18,19].

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