# EVIDENCE FOR THE PRESENCE OF α AMYLASE IN THE CELL MEMBRANE OF BACILLUS AMYLOLIQUEFACIENS

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SUMMARY

The presence of α amylase in the membrane of Bacillus amyloliquefaciens has been investigated. Two forms of the enzyme were found associated with the membrane, a loosely-bound form and a tightly-bound form which required the aid of a detergent for its solubilization. The loosely-bound form of the enzyme, shared a similar electrophoretic mobility with the form tightly-bound to the membrane and a similar sedimentation behavior with the soluble enzyme spontaneously released by the organism into the culture medium. However, the form tightly associated to the membrane differed both in its electrophoretic mobility and its sedimentation behavior from the soluble enzyme found in the culture medium.

The transport mechanism of proteins across the cell membrane is as yet unknown. However, it has been proposed that the extracellular enzymes move across the cell membrane as linear polypeptides while the peptides elongate as they are synthesized in membrane bound ribosomes (1-3). In general, extracellular enzymes behave as hydrophilic proteins (4), however, in Bacillus licheniformis the extracellular enzyme penicillinase has been found in at least two forms: a well characterized hydrophilic form present in the culture medium and a lipophilic form associated with the cell membrane (5-8). Recently, the purification and characterization of the lipophilic form has been achieved (9,10). The findings indicate that the properties of this form of penicillinase differ from those of the hydrophilic form. In addition, a phospholipid is apparently attached to the lipophilic enzyme.

It is known that various water soluble proteins become soluble in organic solvents by virtue of their binding to lecithin and acidic phospholipids (11-14). In various cases, alcohols (11), divalent cations (12) or low pH (13) may be required for effective extraction of the protein into the organic phase. It has been suggested that the change in the solubility properties of the protein upon binding to phospholipids is due to neutralization of its positive charges and its enclosure in a non polar envelope (11,13,14). Indeed, solubilization of a protein in organic solvents can also be achieved with simple synthetic detergents (15).

Considering the above observations, it is possible that the mechanism through which a protein reaches the exterior of the cell, consists in the incorporation of the protein into the cell membrane upon binding to molecules with detergent properties; subsequently, the latter would detach rendering a hydrophilic protein in the exterior of the cell. According to this mechanism a certain amount of the extracellular protein would be present in the cell membrane during the process of secretion. The results presented below indicate that the cell membrane of B. amyloliquefaciens possesses a strongly-bound and a weakly-bound $\alpha$amylase activity, and that these two forms of the enzyme differ from the $\alpha$amylase activity found in the culture media in sedimentation behavior and electrophoretic mobility.

## Materials and Methods

Organism. Bacillus amyloliquefaciens strain P, a highly amylolytic bacillus was obtained from Dr. L. Leon Campbell. All cultures were grown at 30°C on a rotary action shaker at 250 rpm in 2.8 liter Fernbach flasks containing 1000 ml of Antibiotic medium 3 (Difco). Membranes were isolated according to the procedure described by Konings et al. (16) except that 50 mM Tris-HCl, pH 8.0, was used instead of 50 mM potassium phosphate, pH 8.0 and that no EDTA was added. $\alpha$ Amylase activity was assayed as described by Reinderknecht et al. (17). One unit of enzyme

activity is equal to an increment of 0.1 optical density at 595 nm per hour. Proteins were measured by the method of Lowry et al. (18). Linear sucrose gradient centrifugation was carried out as described by Martin and Ames (19). All gradients contained 10 mM potassium-phosphate, pH 8.0, crystalline catalase (Worthington) was used as an internal marker; twelve-drop fractions were collected, providing a total of 18 fractions. Disc electrophoresis was performed through polyacrylamide gels according to the procedure of Davis (20). The substrate Amylopectin Azure was purchased from Calbiochem. The neutral detergent Brij 36T (av. mol.wt. 626) was kindly supplied by Dr. J. Ruiz-Herrera.

## RESULTS AND DISCUSSION

It has been observed that the enzyme $\alpha$ amylase does not accumulate within the cells of Bacillus amyloliquefaciens while the extracellular $\alpha$ amylase activity increases in the culture medium (21). However, according to our hypothesis, certain amount of this enzyme should be present in the cell membrane while the organism is secreting the enzyme, therefore, the presence of $\alpha$amylase activity in the membrane of B. amyloliquefaciens was investigated.

Membrane preparations were obtained from 3 different lots of cells harvested at mid-log phase of growth (65-75 Klett units, employing green filter) and were studied for their content of $\alpha$amylase activity by subjecting them to 3 successive extractions with 100 mM potassium-phosphate, pH 6.6, followed by one or two sonications in the presence of the same buffer and finally by extraction with 10 mM potassium-phosphate, pH 6.6, containing 5 mM Brij 36T. Table 1 shows the total amount of protein and $\alpha$ amylase activity detected in the starting membrane preparations, as well as the total protein and total $\alpha$amylase activity released from the membranes by the different treatments employed. The total amount of

TABLE 1

Comparison of the amount of ∝amylase activity solubilized from *b. amyloliquefaciens* membranes after various treatments.

| Fraction | ∝amylase activity[e] total units | | |
|---|---|---|---|
| | exp 1 | exp 2 | exp 3 |
| Starting membrane preparation[a] | 10.2(2.85)[f] | 9.6(2.65) | 6.0(72.0) |
| First potassium-phosphate buffer extraction[b] | 16.2(0.59) | 6.48(0.34) | 100.0(6.36) |
| Second potassium-phosphate buffer extraction | 18.3(0.08) | 20.5(0.067) | 28.0(4.68) |
| Third potassium-phosphate buffer extraction | 12.9(0.13) | 2.1(0.10) | 9.0(2.4) |
| First sonication[c] | 12.9(0.11) | 47.4(0.10) | --- |
| Second sonication | 0.0(0.12) | 0.0(0.03) | --- |
| Detergent extraction[d] | 56.7(0.14) | 63.7(0.10) | 1228.0(17.2) |
| Residual pellet | 9.0(0.25) | 7.8(0.2) | --- |

[a]Starting membrane preparations were obtained as described under Materials and Methods. For experiments 1 and 2, each membrane preparation was obtained from a separate lot of cells (400 mg wet weight). For experiment 3, membranes were isolated from a third lot of cells (3.6 g wet weight).

[b]For each potassium-phosphate buffer extraction, membranes were suspended thoroughly with a Potter-Elvehjem homogenizer in 9.0 ml of 100mM potassium-phosphate, pH 6.6, and centrifuged at 105,000 x g for 1 hours. Supernatants were assayed for ∝amylase activity and for protein as described under Materials and Methods.

[c]For sonication, membrane pellets obtained after the third potassium-phosphate buffer extraction were suspended thoroughly as above in 2.0 ml of the same buffer and subjected to sonication for 120 seconds at maximum output from a MSE Ultrasonic Power Unit. During sonication, temperature was maintained below 5°C. After sonication, membranes were

enzymatic activity released from the membranes by potassium-phosphate buffer extractions exceeds the activity which can be measured in the starting membrane preparations. This suggests that in the starting membrane preparations either a part of the enzyme is located in the membrane in a situation where it cannot perform enzymatic activity or that some amount of the enzyme present in the membrane is unavailable to the subtrate. However, this type of activity appears to be weakly associated to the membrane as compared to the rest of the enzyme that is liberated from the membrane after detergent treatment. This last type of enzymatic activity could be deeply buried in the membrane.

Since electron microscopy of membrane preparations indicated that these preparations were in vesicular form (data not shown), it was considered that the enzyme being released from the membrane by detergent treatment could be that trapped inside the vesicles and not that integrated in the membrane. In this respect, Table 1 shows that repeated sonication does not release all the enzyme activity from the membranes which have been previously extracted with potassium-phosphate buffer, as would be expected if the detergent released only the enzyme that was trapped in the vesicles. Moreover, when the enzymatic fraction obtained from membranes

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collected by centrifugation at 105,000 x g for 1 hour. Supernatants were assayed for ∝amylase activity and for protein as described under Materials and Methods.

[d]For detergent extraction, membrane pellets were thoroughly suspended with a Potter-Elvehjem homogenizer in 1 ml of 10 mM potassium-phosphate, pH 6.6 containing 5 mM Brij 36T, and incubated for 1 hour at 37°C. Insoluble material was removed by centrifugation at 105,000 x g for 3 hours. Supernatants were assayed for ∝amylase activity and for protein as described under Materials and Methods. The presence of 5 mM Brij 36T did not interfere with ∝amylase assay.

[e]Assay for ∝amylase activity was carried out in 0.1 ml samples of supernatants obtained after each centrifugation and in 0.1 ml (250 ug protein) sample of the starting membrane preparations. Assay of ∝amylase activity present in the residual pellets was carried out after resuspension of the entire pellets in 0.5 ml of 10 mM potassium-phosphate, pH 6.6.

[f]Values in parenthesis show the total amount of protein present in each supernatant or pellet.

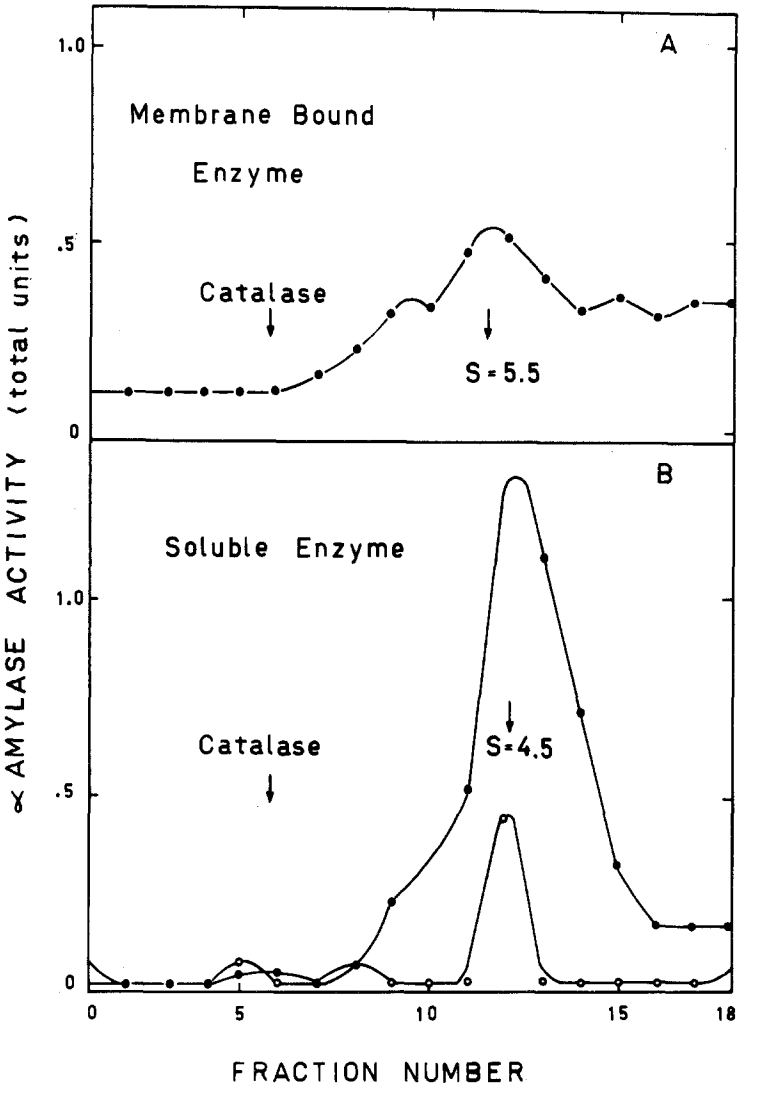


Fig. 1. Sedimentation pattern of ∝amylase activity.
A. Activity solubilized from membranes after detergent treatment.
B. Activity solubilized from membranes after potassium-phosphate buffer extraction (-O--O-), and activity from culture medium (-●--●-). 0.2 ml of 10 mM potassium-phosphate, pH 6.6, containing 5 mM Brij 36T, 1 mg of crystalline catalase and either 2 units of activity solubilized from membranes by detergent, 3 units of activity solubilized from membranes by potassium-phosphate buffer, or 5 units of activity from culture medium were layered on 5 ml linear sucrose gradients (5-20% sucrose). Centrifugation was carried out at 38,000 rpm for 11 hours in a SW-50 rotor in a Spinco model L centrifuge. Each fraction was assayed for ∝amylase activity and for catalase by measuring its absorbance at 405 nm. The approximate sedimentation coefficient for each peak of enzymatic activity was estimated by comparing the position of each peak of activity with the position of the peak of catalase.

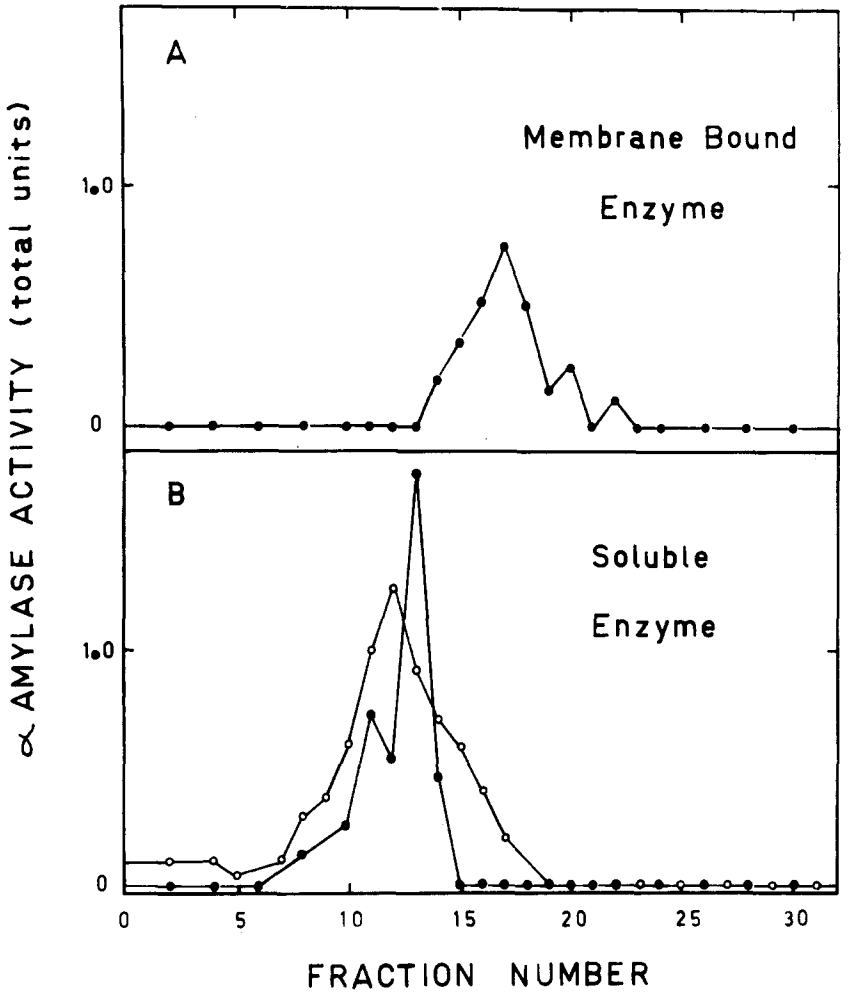


Fig. 2. Electrophoretic analysis of ∝amylase activity.
A. Activity solubilized from membranes after detergent treatment.
B. Activity solubilized from membranes after potassium-phosphate buffer extraction (-O--O-), and activity from culture medium (-●--●-). Sample volumes were 0.1 ml of 10 mM potassium-phosphate, pH 6.6, 5 mM Brij 36T containing either 2 units of activity solubilized from membranes by detergent, 3 units of activity solubilized from membranes by potassium-phosphate buffer, or 5 units of activity from culture medium. Electrophoresis was carried out at 3 ma per column (5 x 70 mm) for 120 min. Gels were sliced into 32 fractions, and each fraction was assayed for ∝amylase activity after eluting the slices with 0.5 ml of 10 mM potassium-phosphate, pH 6.6, for 10 hours.

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by detergent extraction was compared either with the enzymatic fraction released from membranes by potassium-phosphate buffer extraction or with

the enzymatic fraction released spontaneously by the organism into the culture medium, a difference in sedimentation properties was observed. Figure 1 shows the sedimentation profile through 5-20 percent sucrose gradients of the three enzymatic fractions. The detergent-extracted-activity sediments at a faster rate than the other two enzymatic fractions which sediment at the same rate. However, when the three enzymatic fractions were subjected to disc electrophoresis, the detergent extracted fraction and the potassium-phosphate buffer extracted enzyme moved at a faster rate towards the anode than the soluble enzyme found in the culture medium (Fig. 2). These findings indicate that the loosely membrane-bound form of the enzyme which is extracted with potassium-phosphate buffer shares properties with the soluble enzyme fraction present in the culture medium and with the tightly membrane-bound form of the enzyme which is solubilized from the membrane by detergent treatment which apparently possesses either a more compact conformation or a greater negative charge, or both.

Another alternative would be that the $\alpha$amylase activities detected correspond to two different enzymes, one present in the membrane and another always secreted to the culture medium. The loosely membrane-bound form could be the soluble enzyme in the process of secretion at the time of membrane isolation. However, it should be recalled that there is only one structural gene for the enzyme penicillinase in B. licheniformis (23), and that a soluble and a membrane-bound form of the enzyme have been identified and purified (9,10).

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