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Immunochemical Detection of α-Amylase Synthesis by Protoplast Membranes of *Bacillus amyloliquefaciens*

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Abstract

In the cell-free system from *Bacillus amyloliquefaciens* KA-63, protein synthesis was found to proceed exclusively on membrane-bound polysomes (membranes and S-105) and the synthesis was completely inhibited by chloramphenicol and puromycin. In particular, biosynthesis of α-amylase which amounted to 16.2–17.2% of the total protein synthesized could be identified by an immunoprecipitation method, whereas negligible radioactivity was observed in the precipitate in a cell-free system comprising the S-30 fraction.

Similar cell-free systems were prepared from a mutant, AL222, which showed a significantly reduced amount of α-amylase, though the specificity and the immunological properties of the enzyme activity were the same as that of the wild strain. Synthesis of α-amylase was not detected immunologically in the cell-free systems from the mutant.

Introduction

Evidence suggesting that a specific secretory protein is synthesized exclusively on membrane-bound polysomes of endoplasmic reticula has been accumulated through cell-free experiments of mammalian tissues.[1~4] A similar situation might be expected of extracellular enzyme synthesis in bacteria. Cancedda and Schlesinger[5] have shown in *Escherichia coli* that the membrane fraction is enriched with polysomes of alkaline phosphatase, a periplasmic enzyme, in comparison with the cytoplasmic fraction.

But there is little evidence which demonstrates the formation of specific protein directed by endogenous mRNA in cell-free systems of protein synthesis because of fragility of mRNA.[6,7] However, the possible existence of stable mRNAs has been proposed especially for secretory proteins,[8~10] and Hirashima *et al.*[11] have succeeded in demonstrating cell-free synthesis of a specific lipoprotein of the outer membrane of *E. coli* by purified mRNA having a half life of 11.5 min.

In a previous paper, we have reported that a cell-free system of protein synthesis was obtained from exponentially growing cells of *Bacillus amyloliquefaciens* strain KA-63 (formerly *B. subtilis* strain KA-63) and was directed exclusively by endogenous mRNAs bound to membranes.[12] These findings provided us with a suitable system for determining whether secretory protein such as α-amylase is formed preferentially on membrane-bound polysomes. In this paper, the product of the cell-free system directed by membrane-bound polysomes was found by immunological assay to contain a considerable amount of α-amylase.

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Materials and Methods

Bacterial strains *B. amyloliquefaciens* strain KA-63 which had been stored in our laboratory and could accumulate a high level of α-amylase in the medium was used throughout this study. This strain was previously classified as a strain of *B. subtilis*[12]. However, a recent study by Seki *et al.*[13] showed that the strain differs from *B. subtilis* and should be included in *B. amyloliquefaciens*. A mutant, strain AL222, derived from strain KA-63 by *N*-methyl-*N*-nitro-*N*-nitrosoguanidine mutagenesis was also used. Strain AL222 has so far been characterized by accumulation in lower quantities of the same species of α-amylase and protease as the parent strain KA-63.

Preparation of membranes and S-105 extract Cultures were grown in medium containing 50 g of soluble starch, 5 g of peptone, 2 g of yeast extract, 1.3 g of KH_2PO_4, 0.5 g of $MgSO_4 \cdot 7H_2O$, and 0.1 g of $CaCl_2 \cdot 2H_2O$ per liter (pH 7.0) at 30°C in a jar fermenter. Cells were harvested in the exponential growth phase (3 hr culture).

Purified membranes and S-105 extract were prepared by the method of the previous paper at a temperature between 0 and 4°C[12]. Cells (approximately 4.3 g wet weight) harvested from 2,500 ml of broth were washed with 10 mM Tris-HCl buffer, pH 7.6, containing 60 mM NH_4Cl and 10 mM $Mg(CH_3COO)_2$ (standard buffer I) and suspended in 50 ml of the same buffer containing 10% sucrose. The cell suspension was agitated gently with 35 mg of lysozyme (egg white, Kinki Yakult Co. Ltd.) for 60 min at 0°C. The treated cells were harvested by centrifugation at 12,000×*g* for 10 min, and washed once with 50 ml of the standard buffer I containing 10% sucrose and 6 mM β-mercaptoethanol. The protoplasts thus obtained were disrupted osmotically by suspending in 50 ml of 100 mM Tris-HCl buffer, pH 7.6, containing 60 mM NH_4Cl, 10 mM $Mg(CH_3COO)_2$ and 6 mM β-mercaptoethanol (standard buffer II).

The precipitate obtained by centrifugation of the lysate at 30,000×*g* for 10 min was used as crude membranes, and the supernatant was again centrifuged for 20 min under the same conditions. Supernatant thus obtained was used as S-30 extract. The crude membrane fraction was suspended in 25 ml of standard buffer II, and 10 ml aliquots of the suspension were layered over a discontinuous sucrose gradient consisting of 10 ml each of 20% and 60% sucrose in standard buffer I in rotor tubes for an HB-4 swinging bucket rotor of a Sorval RC2-B centrifuge. The tubes were centrifuged at 10,000×*g* for 60 min. The dense layer at the interface was collected by aspiration, suspended in standard buffer II, and purified membranes were obtained by centrifugation at 30,000×*g* for 20 min. S-105 extract was obtained by ultracentrifugation of the S-30 extract at 105,000×*g* for 60 min.

Cell-free system of protein synthesis Cell-free protein synthesis was carried out in a reaction mixture containing 100 mM Tris-HCl (pH 7.6), 10 mM $Mg(CH_3COO)_2$, 60 mM NH_4Cl, 6 mM β-mercaptoethanol, 2 mM ATP, 0.4 mM GTP, 10 mM creatine phosphate, 0.05 mM each of 20 amino acids, 2 μCi per ml of ^{14}C amino acids mixture (chlorella protein hydrolysate having a specific activity of 40 mCi per mg atom C), 40 μg per ml of creatine phosphokinase (Sigma), 1.5~2.4 mg protein per ml of S-105 extract, and 1.0~1.5 mg protein per ml of purified membranes. The reaction was carried out at 30°C. The amount of ^{14}C amino acids incorporated into the protein fraction was determined as described previously.[12].

Sucrose density gradient centrifugation analysis A 0.2 ml aliquot of reaction mixture was layered over 4 ml of a 15~30% linear sucrose gradient on a 0.5 ml 60% sucrose shelf made up in standard buffer I in tubes for Hitachi RPS 40 rotor. The tubes were centrifuged at 20,000 rpm for 4 hr at 4°C. Three-drop fractions were collected from the bottom of the tubes and diluted to 3 ml. The absorbance was measured at 260 nm, and ^{14}C radioactivity in hot trichloroacetic acid(TCA)-insoluble material in 1 ml aliquots was measured as described previously.[12]

Immunoprecipitation of α-amylase synthesized *in vitro* The quantitative determination of α-amylase synthesized in the cell-free system was carried out by an immunoprecipitation method as follows: 0.25 ml of supernatant obtained by centrifugation of the reaction mixture of the *in vitro* protein synthesis at 105,000×*g* for 40 min, and 0.25 ml of purified α-amylase solution (0.5 mg per ml) were mixed and 0.5 ml of anti-amylase serum was added to the mixture. An α-amylase preparation used as carrier was obtained from culture broth (24 hr) of strain KA-63 according to the purification method of Tsujisaka.[14] It was confirmed that the preparation showed a single peak on sodium dodecyl sulfate (SDS) gel electrophoresis. After incubation at 37°C for 60 min, the mixture was left for a few days at 4°C after which the formation of immunoprecipitate was virtually complete. The precipitate thus formed was collected by centrifugation at 3,000×*g* for 10 min and washed at least three times with 0.85% NaCl solution until washing did not cause appreciable liberation of 280 nm absorbing materials. The amount of protein and radioactivity of the resulting immunopercipitate

were determined.

Assay of enzyme activity α-Amylase was determined by the blue value method.[10] The reaction mixture containing 5 ml of 1% soluble starch, 2 ml of 100 mM phosphate buffer (pH 6.0), and 1 ml of enzyme solution was kept at 40°C. One unit of enzyme activity was defined as the amount of enzyme necessary to reduce the blue value of 0.2 ml of reaction mixture in 5 ml of 0.5 mM I_2-KI solution to a critical level (Hitachi spectrophotometer 101, A_{610} 0.4 in a 0.5 cm light path) in 40 sec; 12,500 units corresponded to 1 mg of pure α-amylase protein of strain KA-63. Neutral protease activity was determined by the casein hydrolyzing method at pH 7.6 as described previously.[15]

The amount of protein was determined colorimetrically by the method of Lowry *et al.*[16] with bovine serum albumin (Sigma) as the standard.

Chemicals Antiserum specific for bacterial liquefying α-amylase was kindly supplied by Professor M. Kitagawa. ^{14}C amino acid mixture was purchased from Daiichi Pure Chem. Co.

Results

Protein synthesis in the membrane system The important features of amino acid incorporation into protein by membranes and S-105 extract system are summarized in Table 1. The rate of incorporation in the absence of either S-105 or membranes was negligible. Incorporation was dependent on ATP, GTP, and an ATP-generating system, creatine phosphate and creatine phosphokinase, and practically insensitive to KCN (100 μg per ml). The protein synthesis was not inhibited by actinomycin D but inhibited markedly by chloramphenicol. The above observation could justify the conclusion that the preparations were hardly contaminated, and free from viable cells and protoplasts, as will be discussed later.

Membrane as the site of protein synthesis To elucidate the site of protein synthesis, sedimentation analysis of the complete system for protein synthesis (membranes plus S-105 extract) was carried out. As shown in Fig. 1, a remarkable increase in radioactivity recovered in the hot TCA-insoluble fraction was observed after 30 min incubation in fractions 2–4 which correspond to the boundary phase between 60% and 30% sucrose occupied by membranes.[17] On the other hand, no significant incorporation of the radioactivity was detected in fractions 5–17 in which polysomes are present.[18]

But as shown in Fig. 1B, the peaks of radioactivity coincided with absorbance at

Table 1. Characteristics of ^{14}C amino acids incorporation by cell-free system.

Addition or deletion	Incorp. (%)
Complete	100
−Membranes	0
−S-105	0
−ATP, GTP, phosphocreatine and phosphocreatine-kinase	0
+KCN (100 μg per ml)	108
+Chloramphenicol (100 μg per ml)	15
+Actinomycin D (50 μg per ml)	97

Reaction mixtures were incubated at 30°C for 60 min. The amounts of ^{14}C amino acids incorporated into the hot TCA-insoluble fraction in complete reaction mixture was 5,440 cpm per ml.

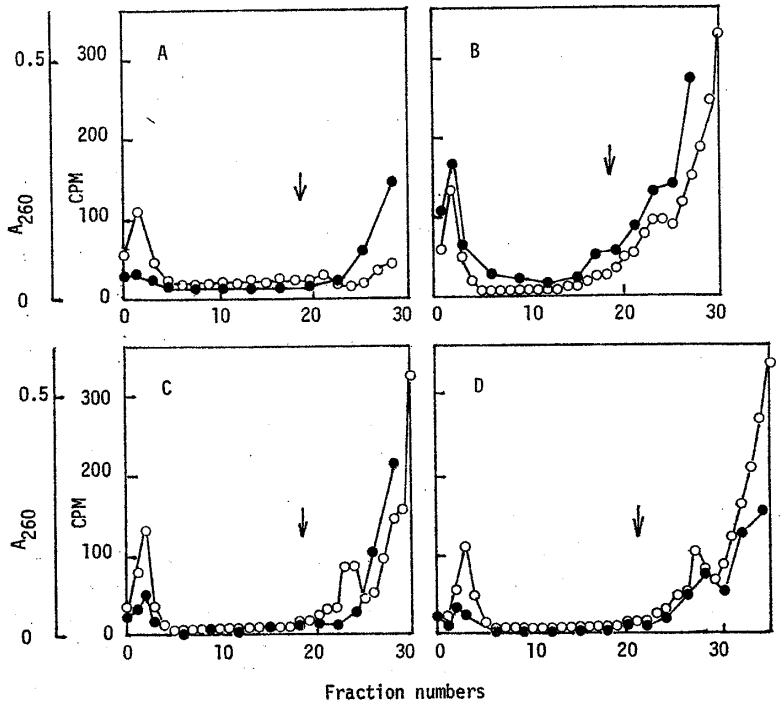

Fig. 1. Sucrose gradient centrifugation analysis of the cell-free system of protein synthesis. Reaction mixtures were incubated at 30°C for 30 min under various conditions indicated below and were centrifuged through a 15–30% linear sucrose gradient on a 60% sucrose shelf at 20,000 rpm for 4 hr. Fractions of three drops were collected from the bottom and absorbance at 260 nm and radioactivity in hot TCA-insoluble material were measured. Reaction mixture used for 0 time control did not contain energy generating components to prevent initiation of the protein synthesis.
A, 0 min reaction; B, 30 min reaction; C, 30 min reaction with 100 μg of chloramphenicol per ml; D, 30 min reaction with 50 μg of puromycin per ml. Fractions 30 and 0 correspond to the top and bottom of centrifuge tube respectively, and the arrow locates 70S ribosomes in the fraction numbers.
Symbols: ○, A_{260}; ●, ^{14}C amino acids incorporated.

260 nm, even though fractions 18–25 were markedly richer in ribosomes and their subunits. On addition of chloramphenicol or puromycin at the beginning of reaction, increase in radioactivity in the membrane fractions as well as in ribosomal fractions was inhibited completely and appearance of free ribosomes decreased (Figs. 1C and 1D). These results, taken with that of Fig. 1B, suggest that translation occurs in membranes and that membrane-bound polysomes are converted to free ribosomes and their subunits during the protein synthesis.

α-Amylase production by AL222 Strain AL222, a mutant which produced less α-amylase than KA-63, showed decreased productivity of protease. Figure 2 shows the time course of production of these enzymes and the growth of strains AL222 and KA-63. In strain AL222, the amount of α-amylase and protease produced after 22 hr cultivation had reached only 5% and 40% of the parental level respectively, in spite of the similar growth patterns.

Immunoassay Anti-amylase serum, kindly provided by Professor M. Kitagawa of the Institute for Cancer Research, Medical School, Osaka University, was prepared from rabbit which was immunized with crystalline liquefying α-amylase (BαA) of *B. subtilis* (Nagase Sangyo Co. Ltd., Japan). To confirm that this anti-amylase serum could cross-react with KA-63 α-amylase as well as BαA, an immunological test was carried out

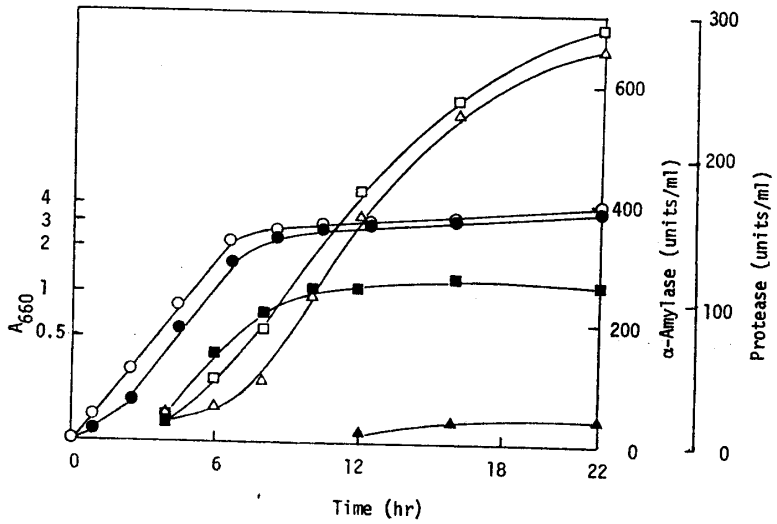

Fig. 2. Time course of α-amylase and protease production by strains KA-63 and AL222. Cells were cultivated at 30°C with shaking and optical density and enzyme activities were measured periodically. Cell density was measured in a 0.5 cm light path at 660 nm. Open symbols, KA-63; closed symbols, AL222. ○ and ●, cell density; □ and ■, protease activity; △ and ▲, α-amylase activity.

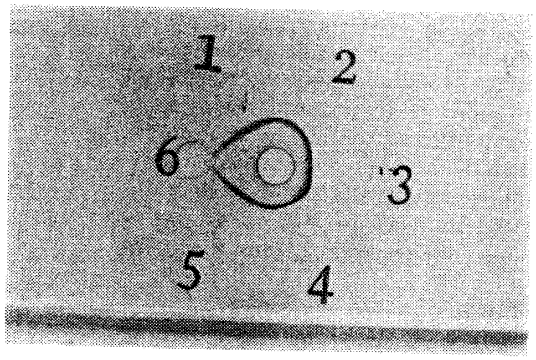

Fig. 3. Double diffusion test for anti-amylase serum. Four ml of 1.5% agar in 50 mM veronal buffer, pH 8.6, was charged on a microscopic slide. Four to five μl of antiserum was placed in the center well, and the same volume of various solutions indicated below was placed in other wells. Diffusion was carried out overnight to form arcs of specific precipitates. The slide was washed with 0.85% NaCl solution for 24 hr to remove the remaining soluble protein, then dried and stained with amido black. 1, 3, 5, KA-63 α-amylase; 2, 4, BαA; 6, cell lysate of strain KA-63.

Figure 3 shows agar gel double diffusion test for BαA and purified KA-63 α-amylase. This anti-amylase serum showed a single precipitation line for both α-amylase, but not for cell lysate of strain KA-63. Almost the same precipitation curves were obtained for both BαA and KA-63 α-amylase in the quantitative precipitation test as shown in Fig. 4 and the amount of antigen-antibody precipitate was maximal at 120 μg of antigen per ml.

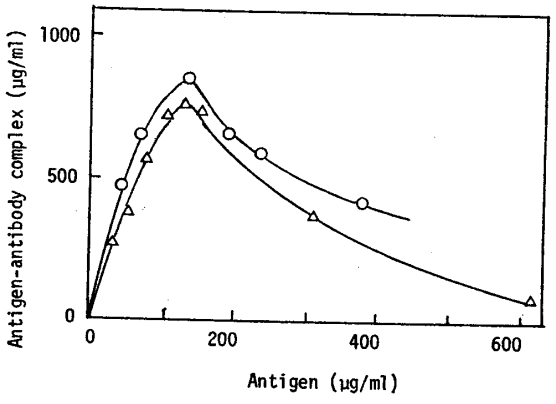

Fig. 4. Quantitative precipitation curves for anti-amylase serum.
A 0.5 ml aliquot of α-amylase solution was mixed with the same volume of antiserum, then incubated at 37°C for 60 min and stored at 4°C for a few days. The amount of protein in immunoprecipitates was determined.
Symbols, ○, KA-63 α-amylase; △, BαA

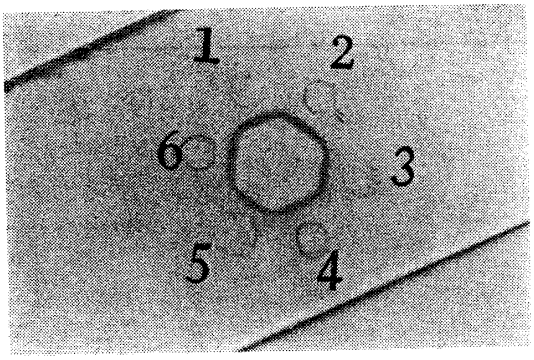

Fig. 5. Double diffusion test for AL222 α-amylase.
Method is as described in Fig. 4.
1, 3, 5, KA-63; 2, 4, 6, AL222

In the antibody-excess region (less than 120 μg of antigen per ml), all of the antigen should be detected in the immunoprecipitate.

This anti-amylase serum also cross-reacted normally with α-amylase produced by strain AL222, when the enzyme solution concentration was increased to 20 times that in the culture filtrate after 22 hr of cultivation (Fig. 5). Figure 6 shows neutralization curves of KA-63 and AL222 α-amylase with anti-amylase serum in the antigen excess regions. No significant difference was noted, implying that α-amylases from both strains have the same immunological properties.

Identification of α-amylase synthesized in the cell-free system Based on these findings, the amount of α-amylase in the products of the cell-free system was assayed immunologically. To avoid possible inclusion of insoluble materials such as ribosomes and membranes in the specific immunoprecipitate, the reaction mixture of *in vitro* protein synthesis was centrifuged at 105,000×*g* for 40 min, and the supernatant was used for the immunological assay. The radioactivity incorporated into hot TCA-insoluble material and immunoprecipitate is summarized in Table 2. The amount of protein synthesized by free polysomes in the cell-free system was determined by the reaction mixture containing S-30 extract instead of membranes and S-105 extract.

In the cell-free system consisting of membranes from strain KA-63, a considerable amount of radioactivity was observed in the immunoprecipitate, corresponding to 16.2~17.2% of the total radioactivity incorporated into the hot TCA-insoluble material (Table 2 Exp. 1 and 2). Further, when membranes obtained from cells producing α-amylase at the maximal rate (6 hr culture) were employed in the cell-free system, the recovery of radioactivity in the immunoprecipitate was 63.2% of the total incorporation. However, in the S-30 extract system, no more than 0.7~1.7% of the total radioactivity in the hot TCA-insoluble material was observed in the immunoprecipitate (Table 2).

Radioactivity was not observed in the immunoprecipitate of either the membrane or S-30 cell-free system from exponentially growing cells of AL222 despite the fact that these cell-free systems had sufficient activity to incorporate ^{14}C amino acids into hot TCA-

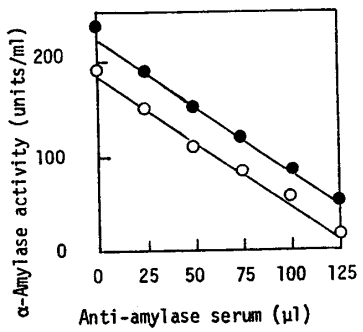

Fig. 6. Neutralization of α-amylase with anti-amylase serum.
A 0.5 ml aliquot of enzyme solution containing 250 units was incubated with various amounts of the antiserum at 40°C for 60 min. The activity remaining was determined.
Symbols: ○, KA-63, ●, AL222

Table 2. *In vitro* synthesis of α-amylase.

Strain	Exp.	System	The amount of radioactivity in hot TCA-insoluble material (A) (cpm/ml)	The amount of radio activity in immunoprecipitate (B) (cpm/ml)	Recovery (B/A) (%)
KA-63	1	Membrane	15,080	2,600	17.2
		S-30	49,810	360	0.7
	2	Membrane	9,890	1,600	16.2
		Membrane*	4,050	2,560	63.2
		S-30	35,780	600	1.7
AL222	3	Membrane	18,100	ND**	—
		S-30	59,360	ND**	—

The amount of radioactivity in hot TCA-insoluble material was measured after 60 min incubation at 30°C in complete reaction mixture. Reaction mixture of S-30 cell-free system contained 1.4–1.5 mg protein per ml of S-30 extract instead of membranes and S-105 extract.
* Membranes from 6 hr culture cells in maximal producing phase of α-amylase.
** Not detectable.

insoluble material (Table 2 Exp. 3). These results, taken with the fact that strain AL222 has reduced productivity of α-amylase, suggest that radioactivity in immunoprecipitate represents the amount of α-amylase in the products of the cell-free system.

Discussion

It was evident from Table 1 that amino acid incorporation into protein in this system was not due to the presence of contaminating intact cells and protoplasts. Firstly, neither membranes alone nor S-105 extract incorporated appreciable quantities of amino acids: in fact, both fractions were required for the protein synthesis. Secondly, the cell-free system of membranes plus S-105 extract was virtually inactive when ATP, GTP and ATP-generating system were omitted from the complete reaction mixture. Furthermore, amino acid incorporation was practically insensitive to KCN, and neither intact cells nor protoplasts were visible microscopically in the reaction mixture.

It was shown by sucrose density gradient centrifugation that increase in radioactivity occurred in membrane fractions during incubation and was inhibited by chloramphenicol and puromycin (Fig. 1), indicating that radioactivity detected in hot TCA-insoluble fraction represents the amount of polypeptide chains synthesized *de novo*. On the other hand, no significant increase in radioactivity or absorbance at 260 nm was seen in fractions 5–17 which would contain polysomes if present. This observation suggests that polysome fractions do not participate in the protein synthesis and that only a small amount of polysomes was liberated from membranes in the process of protein synthesis. However, in fractions 19–25 containing monoribosomes and their subunits, radioactivity and absorbance at 260 nm increased during incubation, coinciding with the fact that dissociation of ribosomes resulted in cessation of protein synthesis in membrane cell-free system.[12] Increase in radioactivity in these fractions might be attributed to polypeptide chains released from membranes after *de novo* synthesis. These results demonstrate that protein synthesis occurs exclusively in the membrane fraction in this cell-free system.

It seems significant to refer to properties of strain AL222 and its α-amylase, before discussing immunological determination of α-amylase synthesis *in vitro*. As shown in Fig. 6, α-amylase produced by strain AL222 was identical to that of strain KA-63 in the

immunological neutralization curves for anti-amylase serum, indicating that both enzymes had the same specificity toward the anti-serum. This suggests that α-amylase in the mutant is unlikely to carry mutation at the structural gene. This idea is also supported by the fact that simultaneous degeneration of both activities of α-amylase and protease was observed in strain AL222. Mutations which affect productivities of both α-amylase and protease simultaneously have been reported in *B. subtilis* from several laboratories. One of them, the amy B gene, controls flagellation as well as productivities of α-amylase and protease.[19] Similarly, the *pap* mutation, which stimulates production of α-amylase and protease simultaneously, is lacking one protein component in its membrane as demonstrated by SDS gel electrophoresis.[20] These genes are thought to be related to the secretory mechanism. In strain AL222, protein composition of membrane was also altered in SDS gel electrophoresis (unpublished results). These results imply that the protein synthesizing site for extracellular enzymes is localized exclusively in the membranes.

In this experiment, incorporation of radioactive amino acids into the immunoprecipitate was taken as proof for *in vitro* synthesis of α-amylase, because it was difficult to detect enzymatic activity of α-amylase in the cell-free product. So it is uncertain whether a biologically active enzyme protein as well as full protein chain is synthesized in this reaction. However, as shown in Table 1, more than 15% of total incorporation by membranes of strain KA-63 was of α-amylase origin, while this value decreased to one-tenth in protein synthesized by cytoplasmic polysomes. This value may be accounted for by the possibility that S-30 extract contains polysomes which were originally bound to the membranes and released during the preparation procedure. Furthermore, in the membrane system obtained from cells of strain KA-63 in the maximum α-amylase producing phase, 60% of the total protein synthesized *in vitro* was of α-amylase. These results suggest that α-amylase is synthesized on membrane-bound polysomes and that mRNA specific for α-amylase is accumulated in the membrane fraction, escaping from degradation in the course of cell growth.

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