THE SYNTHESIS OF α-AMYLASE BY A CELL-FREE SYSTEM FROM BACILLUS SUBTILIS

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It has been well known that some strains of B. subtilis produce a large amount of α-amylase, ribonuclease and protease in the culture medium, exclusively in stationary phase of the cell growth. Extensive studies to clarify the site and mechanism of the synthesis of these extracellular enzymes have been done in this laboratory (Nomura et al, 1957). However, in order to get more precise information, it needs to be established that there is a system which can synthesize one of these extracellular enzymes at the cell-free level. The present preliminary report deals with the demonstration of the synthesis of α-amylase by a cell-free system from B. subtilis and some properties of the preparation.

EXPERIMENTAL

B. subtilis strain K was grown aerobically at 30°C in a medium containing citrate and glutamate as main carbon and nitrogen sources. The cells were harvested by refrigerated centrifugation in the stationary phase after the α-amylase activity had reached 25-30 units* per ml of culture. The cells were then washed once with cold 0.01 M Tris(hydroxymethyl)aminomethane buffer, pH 7.4, containing $MgCl_2$ (0.01 M) and EDTA (0.005 M), twice with cold "standard buffer": Tris (0.01 M) buffer, pH 7.4, containing $MgCl_2$ (0.01 M), KCl (0.1 M) and β-mercaptoethanol (0.003 M). The packed cells were rapidly frozen by dryice-alcohol after washing and stored at -25°C.

* One unit of α-amylase activity was defined according to Hagihara (1954).

In order to get an active cell-free preparation, the frozen cells were suspended in "standard buffer" containing sucrose (0.25 M) and polyvinyl sulfate (1.25 mg/ml), and disrupted by being passed once through a French pressure cell. The extract was centrifuged at 12,500 x g for 20 min, and again at 17,500 x g for 30 min to remove cell membrane or cell wall fraction. The supernatant was then centrifuged at 105,000 x g for 90 min to sediment small particles. The supernatant soluble fraction was dialyzed for 3 hours against "standard buffer" (105S). The particle fraction was washed with "standard buffer" containing sucrose and centrifuged at 105,000 x g for 90 min (105P). Soluble and particle fraction from logarithmic phase cells when no α-amylase production was detectable were also prepared in the same way (105S-L, 105P-L). All manuplations were carried out at $0^{\circ}C$-$5^{\circ}C$.

Chemical composition of the particle fraction was shown to be composed of 55 weight % of protein, 45 weight % of RNA and trace amounts of DNA (less than 0.1 %).

RESULTS AND DISCUSSIONS

When 105P was incubated with 105S in the presence of ATP, ATP generator, amino acids and cofactors as described in the legend of Table 1, an increase of about 50 % in α-amylase activity over the initial amount of the enzyme present in the preparation was observed with 40 minutes incubation. In Fig. 1 the increase in α-amylase activity is presented as a function of incubation time.

The amount of α-amylase increased by this system was about 0.2 μg per mg of particle RNA present. A fairly sharp pH optimum was observed with maximum increase at 7.5. The cell membrane or cell wall fraction was also active, but less efficient than 105P.

In Table 1 (next page) are presented some characteristics of α-amylase synthesis by cell-free system. The increase in α-amylase activity was dependent upon addition of ATP and ATP generator, and to a lesser extent, upon addition of GTP, CTP and UTP. Omission of KCl or sucrose,

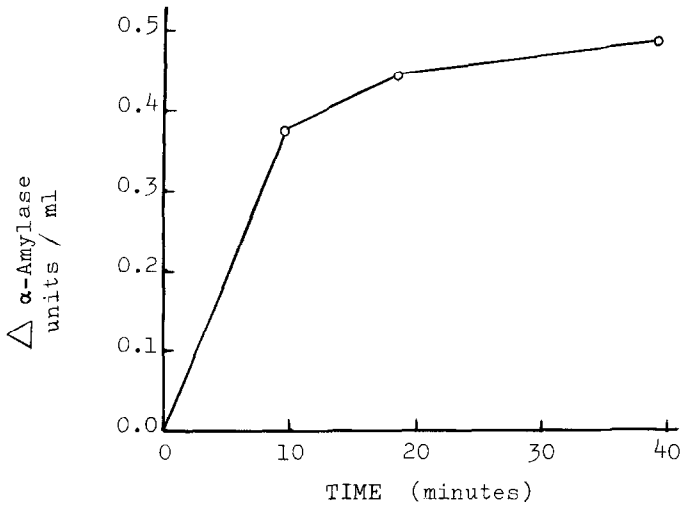

Fig. 1. The increase in α-amylase activity as a function of incubation time. The reaction mixture and assay method is presented in Table 1. Particle fraction (105P) contained 1.25 mg RNA. 0 time α-amylase activity was 0.98 units.

presumably stabilisers of the particles in this case, resulted in almost complete loss of increase in α-amylase activity. Glycerol could be substituted for sucrose. Ca^{++} ion was also required for the maximum increase of the activity. The Factor, which was isolated from nucleic acid fraction of this organism and stimulates remarkably the production of α-amylase and other extracellular enzymes in vivo (Yoshikawa and Maruo 1960), had little or no effect in this system. Omission of soluble fraction reduced the capacity of this system about one half.

The increase in α-amylase activity was inhibited almost completely by 2.5 μg/ml of RNase and 50 μg/ml of chloramphenicol. When 5.0 μg/ml of DNase was added, approximately 30-60 % of the inhibition was observed. Increasing the concentration of DNase 10 fold beyond this did not increase the inhibitory effect of DNase. The effect of DNase as well as that of the mixture of GTP, CTP and UTP suggest that DNA may play an assential role in α-amylase synthesis by B. subtilis, presumably through messenger RNA synthesis. Preliminary experiments showed that addition of DNA from

Table 1

Characteristics of α-amylase synthesis by the cell-free system

			Δ α-Amylase units / 40 min
Complete system			0.45
"	minus	ATP and generator	0.12
"	"	GTP, CTP and UTP	0.24
"	"	KCl	0.05
"	"	$CaCl_2$	0.28
"	"	$MnCl_2$	0.33
"	"	L-amino acids	0.35
"	"	sucrose	0.11
"	"	Factor	0.39
"	"	soluble fraction (105S)	0.25
"	plus	RNase (2.5 μg)	0.07
"	"	DNase (5.0 μg)	0.17
"	"	chloramphenicol	0.10

The reaction mixture contained the following in μmole/ml; Tris(hydroxymethyl)aminomethane 100, pH 7.4; $MgCl_2$ 10; KCl 100; $CaCl_2$ 1.0; $MnCl_2$ 0.8; ATP 5.0; creatinephosphate 5.0; creatinephosphokinase 50 μg; GTP,CTP and UTP 0.1 each; 19 L-amino acids (α-amylase equilibrated) 5.0; sucrose 250; Factor (leucine equivalent) 30 μg; β-mercaptoethanol 1.0; particle fraction 1.25 mg RNA; soluble fraction 0.1 mg RNA. Samples were incubated at 35°C for 40 min, and the reaction was terminated by chilling the samples and adding RNase (5 μg) to each tube. 0 time α-amylase activity was 0.98 units. α-Amylase activity was assayed by the method of Hagihara (1954).

B. subtilis and RNA polymerase fraction from E. coli (Chamberlin and Berg 1962) to this system stimulated the increase in α-amylase activity.

The results of the experiment with particles (105P-L) and soluble fraction (105S-L) from logarithmic phase cells are shown in Table 2. It is clear that the highest increase in α-amylase activity was observed only when 105P was incubated with 105S, but there is no increase in the enzyme activity when 105P-L was incubated with 105S or 105S-L. From

these results, it seems likely that not only soluble fraction but the particle fraction are organized as to permit the synthesis of α-amylase in stationary phase of the cell growth.

Table 2

Specificity of particle and soluble fraction from the cells of different phase for α-amylase synthesis

Particle fraction	Soluble fraction	Δ α-Amylase units / 40 min
105P	——	0.55
——	105S	< 0.05
105P	105S	1.02
105P	105S-L	0.49
105P-L	105S	< 0.05
105P-L	105S-L	< 0.05

The reaction mixture and assay method is the same as in Table 1. 105P, 105P-L, 105S and 105S-L contained 1.58, 1.62, 0.35 and 0.30 mg RNA, respectively.

In summary a cell-free system for α-amylase synthesis was obtained from B. subtilis. From the results of the experiments with this system it was concluded that; firstly, small particles rich in RNA serve as the site for the synthesis of the extracelluar α-amylase by B. subtilis, secondly, DNA existed in soluble fraction may participate in the synthesis of α-amylase.

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REFERENCES

Chamberlin, M., and Berg, P., Proc. Natl. Acad. Sci., 48, 81 (1962)

Hagihara, B., Ann. Rep. Scient. Works, Fac. Sci. Osaka. Univ. 2, 35 (1954)

Nomura, M., Hosoda, J., Yoshikawa, H., and Nishimura, S., Proc. Int. Symposium on Enzyme Chem. (Tokyo and Kyoto), p. 359 (1957)

Yoshikawa, H., and Maruo, B., Biochim. et Biophys. Acta, 45, 270 (1960)