STIMULATION OF AMYLASE FORMATION BY AN AMINE FROM *BACILLUS SUBTILIS*

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SUMMARY

An active factor which stimulates amylase formation by *Bacillus subtilis* has been extracted from *B. subtilis* cells and purified. The factor resembles alkyldiamines or polyalkylamines in its chromatographic behavior, its reaction with ribonucleic acids and other types of reactions.

Authentic samples of amines such as putrescine, cadaverine, spermidine and spermine have been found to stimulate amylase formation at high concentrations. None of them however is identical with the factor.

The factor also stimulates the production of other exo-enzymes of *B. subtilis*, *e.g.*, proteases, ribonuclease, and γ-glutamyltransferase. Moreover it stimulates the turnover of RNA and polyphosphate in the cells to a greater extent than it stimulates enzyme production.

In vitro experiments showed that in the presence of the factor, RNA associates to larger molecules than those of the original RNA.

The chemical structure of the factor and its biological functions are discussed.

INTRODUCTION

It has been reported previously that the formation of amylase from resting cells of *Bacillus subtilis* was stimulated by a ribonucleic acid (RNA) fraction prepared from cells of the same strain[1].

Treatment of the RNA fraction with ribonuclease (RNase) did not abolish the stimulating activity, and the acid soluble fraction of the RNase digest (R-Hps) was used as an active crude factor. The activity of this factor was destroyed by acid or alkaline hydrolysis, and preparations obtained from the cells which produce small amount of amylase were also inactive. From these experimental results, the active moiety of the factor was presumed to be either a specific oligonucleotide or a specific substance attached to nucleic acid.

Further fractionation of R-Hps was carried out. During the purification of the factor extensive elimination of RNA had no effect on the activity of the factor and an almost pure active material was isolated. This material was basic and had a strong affinity for RNA.

There is some evidence that basic materials, *i.e.*, basic amino acids or biological

amines[2,3], may play some essential role in the protein synthesis especially by interaction with acidic materials such as RNA and deoxyribonucleic acid (DNA).

In the present paper the procedure for purification, the chemical nature of the purified material, and some of its biological functions are presented. The data obtained suggest that the active moiety is a derivative of an alkyldiamine or a polyalkylamine.

METHODS

Strain

Bacillus subtilis strains, H, N, and K were used in this investigation. Strain H was mainly used as a source of active factor and strain N was used for the assay of the active factor.

Media and culture

The media and the cultures of the cells used both for the preparation of the factor and its assay have already been reported in an earlier paper[1]. The preparation of an assay system for the active moiety has been also described previously[1].

Assay procedure

Amylase was assayed by the method of HAGIHARA[4]. Protease was assayed by the method of CHARNEY AND THOMARELL[5]. RNase was assayed by the method of REDDI[6]. γ-glutamyltransferase was determined by measuring the optical density of the color which is formed on reaction of γ-L-glutamylhydroxamate with $FeCl_2$ at 540 mμ in a Beckman-type spectrophotometer.

Analytical methods

Protein and RNA were fractionated by the method of SCHMIDT-TANNHAUSER[7]. The total nucleic acid content was measured from the u.v. absorption, assuming that the optical density at 260 mμ of nucleic acid solution containing 1 mg/ml is 22 [8]. The protein content was determined by FOLIN's reagent[9]. Inorganic phosphate was determined by ALLEN's reagent[10]. The radioactivity was determined by a conventional Geiger-Müller counter with an end window.

RESULTS

Purification of the active factor

The preparation of R-Hps has already been reported[1]. The procedure for the further purification of the active factor is presented in Fig. 1, and the analytical data obtained in different stages of the purification are shown in Table I.

When RHps was added at pH 7.3 onto the Dowex-1 (Cl) column and eluted with water, more than 90 % of the biological activity was eluted out, although 95 % of the u.v. light absorbing materials contained in the RHps preparation remained in the column. It is clear, therefore, that extensive elimination of the ribonucleic acid components had no effect on the activity of the factor. The non-RNA nature of the active material is very similar to that of the incorporation factor described recently by GALE AND FOLKES[11].

Activated charcoal was added to the eluate from Dowex-1 at 20 mg/ml which was then filtered through sintered glass after stirring for 15 min. Approximately 50 %

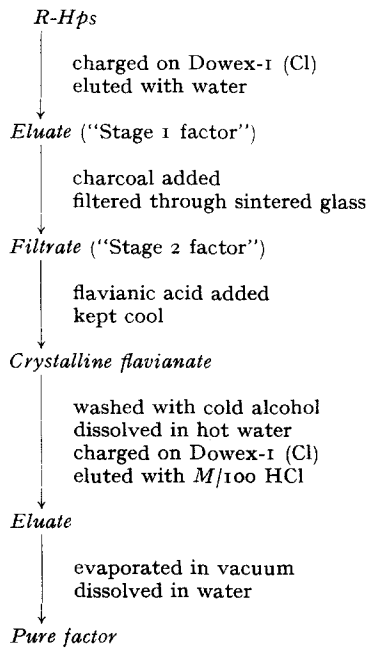

Fig. 1. Fractionation diagram for active factor.

TABLE I

ANALYTICAL DATA FOR VARIOUS FACTOR PREPARATIONS

Various factor preparations derived from the same amount of original preparation, Hp, which contained 1 mg/ml RNA were analysed. The results of the analysis of similar samples were in agreement with the data presented here.

Stages of purification	*RNA (mg/ml)*	*Protein (mg/ml)*	*Ninhydrin value* (mg/ml)*	*Polysaccharide** (mg/ml)*	*Dry weight (mg/ml)*
Hp	1.0	1.3	0.18	0.38	
R-Hps	0.63	0.18	0.13	0.05	1.9
Stage-1 factor	0.028		0.096	0.035	1.0
Stage-2 factor	0.00	0.00	0.064	0.00	0.3
Pure factor	0.00	0.00	0.016	0.00	0.05

* The amounts are shown as leucine equivalents.
** Polysaccharide is measured by anthron reagent and is shown as glucose equivalents.

of the total activity was recovered in the filtrate. By this procedure the remaining nucleic acid components and oligosaccharides were exclusively eliminated. Paper-chromatography of the charcoal-treated factor preparation (stage 2 factor) was carried out. The charcoal filtrate was concentrated and chromatographed on Toyoroshi filter paper No. 51, 10 × 40 cm, in the three solvent systems shown in Table II. In each system a single spot was obtained which reacted with ninhydrin reagent. After the chromatograms had been developed and dried, they were cut into pieces and were eluted with water to test for biological activity. Both the active fraction and the ninhydrin positive spot were found in the same region. Thus it is supposed

that the material which is reactive with ninhydrin is the active moiety. By paper electrophoresis it was shown that the active material was positively charged at pH 6.5 (*cf.* Table II).

Further purification was attempted by precipitation with flavianic acid when yellow needle-like crystals were easily obtained from cold aqueous solution. The crystals were washed twice with cold absolute alcohol and dissolved in hot water.

TABLE II

CHEMICAL NATURE OF THE FACTOR: COMPARISON WITH SOME AMINES

Factor and amines	*RF on paper chromatography**			*Mobility on paper electrophoresis** (cm)*	*Biological activity*** (% activation)*
	Solvent I	*Solvent II*	*Solvent III*		
Stage 2 factor	0.05–0.15	0.42–0.50	0.51–0.57	—3.8 – —4.8	390
Putrescine	0.08–0.12		0.57–0.60	—8.3	50
Cadaverine	0.12–0.18	0.30–0.50	0.60–0.63	—8.0	135
Spermidine	0.00–0.04		0.32–0.40	—4.8	151
Spermine	0.00–0.08	0.28–0.60	0.17–0.23	—4.8	240

* All the paper chromatography was carried out by the ascending method at room temperature. Solvent I: butanol–acetic acid–water = 4:1:1 (by volume); Solvent II: butanol saturated with concentrated NH_4OH; Solvent III: methylcellosolve–propionic acid–water = 75:15:15 (by volume).

** Conditions of electrophoresis: Toyoroshi No. 51, 2 × 15 cm, *M*/10 citric acid buffer, pH 6.5, 200 V, 2.7 mA, for 2 h.

*** Biological activity was assayed in resting cells.

The solution was then charged on Dowex-1 (Cl) column and eluted with *M*/100 HCl. About 30% of the ninhydrin positive material originally contained in the stage 2 factor was eluted and the flavianic acid remained on the column. After the HCl had been evaporated in a vacuum the residue was shown to stimulate amylase formation.

Chemical nature of the factor

As the yield of the pure factor is very small, stage 2 factor is used throughout the following experiments.

The factor showed a positive ninhydrin reaction but the color reactions for hexoses, ribose, hexoseamine, aromatic amines, histidine, and arginine were all negative. The factor is basic and combines with inorganic phosphate, flavianic acid and ribonucleic acid (*cf.* Fig. 3).

It was shown in Table II that the factor has the properties of polyalkylamines or polymethylenediamines judging from its behavior on both paper chromatography and paper electrophoresis using the appropriate solvent systems of HERBST *et al.*[12]. We have not yet found the natural amine which is identical with the factor.

Effect of alkyldiamines and polyalkylamines on amylase formation

As shown in Table II some natural amines, putrescine, cadaverine, spermidine, and spermine, showed a stimulating effect on amylase formation: spermine has the highest activity, almost 2/3 of that of the purified factor from *B. subtilis*. The concentration of the amines which is necessary to show their activity, however is five or ten times higher than that of the factor. The saturation curve of the effect of the

factor on amylase and protease is presented in Fig. 2. These results strongly support the idea that the active material is a polyalkylamine, alkyldiamine, or one of their derivatives.

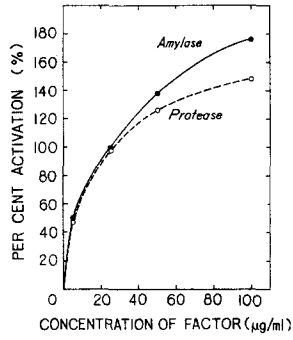

Fig. 2. Saturation curve of the effect of factor on amylase and protease formation.

$$\text{Percent activation} = \frac{(\Delta \text{enzyme activity with addition} - \Delta \text{enzyme activity without addition}) \times 100.}{\Delta \text{enzyme activity without addition}}$$

Resting cells were incubated for 3 h. with the addition of various amounts of the factor, and the increase of enzyme activity was measured.

Biological functions of the factor

Stimulation of enzyme formation: Resting cells of *B. subtilis* strain N form exoenzymes, protease, and ribonuclease together with amylase[1,13,14]. Also resting cells of strain K form the exoenzyme γ-glutamyltransferase. As shown in Table III the formation of these exoenzymes is stimulated by the factor. NISHIMURA found that strain N has ribonuclease inside the cell which is different in various respects from the exoenzyme ribonuclease[15]. In resting cells, however, neither an increase of internal ribonuclease nor the effect of the factor on the formation of the enzyme was observed.

Reaction of factor with RNA in vitro: As mentioned above, the factor gives an insoluble complex with inorganic phosphate in water. It was also shown that the factor combined with RNA. Commercial yeast RNA was dissolved (1 mg/ml) in $M/30$ tris(hydroxymethyl)aminomethane (Tris) buffer (pH 7.3). When the RNA solution was added to two volumes of 99.5 % alcohol in ice water, precipitation of RNA was

TABLE III

THE EFFECT OF THE FACTOR ON THE FORMATION OF EXOENZYMES

The amounts of amylase and protease were calculated by comparing the activities of the enzymes in the culture aliquots with the activities of the crystalline enzymes. Ribonuclease was calculated by comparing with purified ribonuclease. γ-glutamyltransferase was determined by measuring the optical density at 540 mμ of the color of γ-glutamylhydroxamate assuming that a 1.0 increase in O.D.^{540} was 1 unit.

Enzymes	*Increase of enzyme activity/3 h (μg/ml)*		*Per cent activation (%)*
	No addition	*+ Factor*	
Amylase	17	45	170
Protease	8	32	300
Ribonuclease	0.04	0.12	200
γ-Glutamyl transferase	0.2	0.55	170

not observed. But when alcohol was added to the RNA solution in the presence of low concentrations of the factor, heavy precipitation occurred at once. Alcoholic precipitation of 0.1 % RNA solution in the presence of various amounts of the factor, cadaverine, and spermine were examined and the results are presented in Fig. 3 and Table IV. It is shown that the factor as well as cadaverine and spermine at low concentrations combined with RNA to produce complexes insoluble in 66 % alcohol. The factor has the strongest affinity for RNA.

TABLE IV

PRECIPITATION OF RNA IN THE PRESENCE OF THE FACTOR

The experimental method and the reaction mixture are shown in Fig. 3. The amount of the factor soluble in 66 % alcohol was measured with ninhydrin and calculated in leucine equivalents. The amount of the factor shown in the last column was calculated by the difference between the amount of the factor added and that which was soluble in 66 % alcohol.

Amounts of the factor added (μg)	*Per cent of RNA soluble in 66 % alcohol* (%)	*Amounts of the factor soluble in 66 % alcohol* (μg)	*Amounts of the factor precipitated with RNA* (μg)
0	100	0	0
7.5	102		
37.5	7	< 5	> 32.5
75	2	< 5	> 70
375	2	266	109
750	2	578	172

A change of molecular weight of RNA in the solution in the presence of the factor was assumed and the sedimentation velocity of the RNA solution (5 mg/ml) was determined by an analytical Spinco E type ultracentrifuge in the presence and absence of the factor (450 μg/ml). The results showed that the sedimentation constant of RNA at 20° is 2.7 in the absence of the factor and is 4.2 in the presence of the factor. This means that the size of the molecule of RNA is, in the presence of the factor, approximately twice the size of the original one.

Effect of the factor on the metabolism of RNA in the cell: The effect of the factor on the incorporation of ^{32}P-labeled inorganic phosphate into RNA in the cell was examined.

Resting cells of *B. subtilis* strain N suspended in *M*/30 phosphate buffer (pH 7.3) were shaken at 30° for 60 min, and then ^{32}P-labeled inorganic phosphate was added. Aliquots were taken at various times and a RNA fraction was separated by the method of SCHMIDT-TANNHAUSER[7].

The RNA content of the cell decreased gradually during the incubation and after 60 min it was 70–80 % of the original amount. The rate of incorporation of ^{32}P into RNA is very slow. It was seen that after 60 min less than 1 % of the total phosphate in the RNA was labeled with ^{32}P.

As shown in Fig. 4 the factor stimulated the incorporation reaction. Both the incorporation of ^{32}P into control RNA and the stimulation of the factor were inhibited by the addition of 2,4-dinitrophenol ($1 \cdot 10^{-4}$ *M*). In many experiments the stimulating effect of the factor on ^{32}P incorporation runs parallel with that on enzyme formation.

But the former is always larger (5–10 times stimulation) than the latter (2–3 times stimulation).

Effect of the factor on the incorporation of ^{32}P into polyphosphate. Preliminary studies by paper chromatography showed that the greater part of ^{32}P incorporated into the RNA fraction is in phosphate compounds other than the four nucleotides. These unknown phosphate compounds are supposed to be polyphosphates. Therefore, the cells were incubated with ^{32}P-labeled inorganic phosphate for 2 h and fractionated

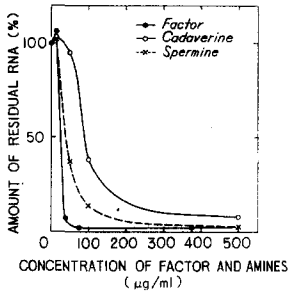

Fig. 3. Precipitation of RNA in the presence of factor and amines. 1 mg yeast RNA was dissolved with various amounts of factor and amines in 1 ml $M/30$ Tris buffer (pH 7.3). After incubation at 0° for 1 h 2 volumes of 99.5% alcohol were added and the precipitate centrifuged. The RAN content in the supernatant fraction was measured.

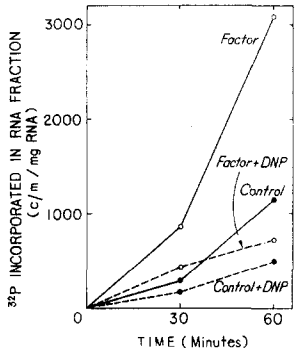

Fig. 4. Incorporation of ^{32}P-labeled inorganic phosphate into RNA fraction. Incubation mixture: washed cells suspended in $M/15$ phosphate buffer (pH 7.3), 1.5 ml; addition of ^{32}P-labeled inorganic phosphate, $6.5 \cdot 10^5$ counts/ml; 2,4-dinitrophenol, $1 \cdot 10^{-4}$ M; factor, 100 μg/ml as leucine equivalents; total volume = 3 ml.

TABLE V

THE INCORPORATION OF ^{32}P-LABELED INORGANIC PHOSPHATE INTO RNA AND POLYPHOSPHATE FRACTIONS

The methods of incubation and fractionation of RNA and polyphosphate are described in the text. Incubation mixture: washed cells suspended in $M/15$ phosphate buffer (pH 7.3), 1.5 ml; addition of ^{32}P, $5 \cdot 10^6$ counts/ml; factor, 100 μg/ml; total volume 3 ml.

	Time 0 h	*After 2 h*		*Per cent activation* (%)
		Control	*+ Factor*	
Amylase activity (μ/ml)	0.6	1.1	2.4	260
RNA fraction				
Amounts of RNA (mg/ml)	0.27	0.11	0.11	
Total counts (counts/ml)	20.4	270	1070	
Specific activity (counts/mg RNA)	76	2450	9700	296
Polyphosphate fraction				
Amounts of polyphosphate (mg/ml)	0.013	0.015	0.015	
Total counts (counts/ml)	451	3430	27000	
Specific activity (counts/μg poly-p)	34.6	228	1800	690
$\frac{\text{poly-p}}{\text{RNA + poly-p}} \times 100$*	95.8	92.7	95.2	

* The ratio of total counts of the fractions.

as follows: the cells were washed with cold 5 % perchloric acid (PCA) and then washed with a hot ethanol–ether mixture. The insoluble fraction was then extracted with 1 *M* NaCl for 60 min in a boiling water bath. RNA was precipitated with alcohol from the NaCl extract. The remaining fraction, insoluble in hot NaCl, was extracted with 5 % trichloroacetic acid for 15 min in boiling water. The extracted fraction contained a small amount of u.v. light absorbing material, and a large amount of inorganic phosphate was released after hydrolysis with 1 *M* HCl for 10 min in boiling water. This fraction represented the polyphosphate fraction.

The incorporation of ^{32}P into both the RNA and polyphosphate fractions was examined separately, and one example of this experiment is presented in Table V. Above 90 % of ^{32}P incorporated in the RNA fraction obtained by the method of SCHMIDT-TANNHAUSER is in fact incorporated into the polyphosphate fraction. It was also shown that during 2 h 0.3 % of total phosphate in RNA and 3 % of total phosphate in polyphosphate were labeled with ^{32}P.

The stimulating effect of the factor is predominant on the incorporation of ^{32}P into the polyphosphate fraction.

DISCUSSION

The purified factor which stimulates amylase formation by *B. subtilis* shows the following chemical properties. 1. The active purified factor is reactive only with ninhydrin, and the color developed on paper is red. The rate of reaction with ninhydrin in solution is very slow. 2. It is strongly basic and combines with inorganic phosphate and flavianic acid. 3. On paper chromatography it runs fast in basic solvents and very slowly in acidic solvents. These chemical observations are the characteristic features of alkyldiamines and polyalkylamines. The biological activity of the factor is destroyed by acid and alkali. On the other hand biological alkyldiamines and polyalkylamines, such as 1,3-propandiamine, putrescine, cadaverine, spermidine, and spermine are stable in both acid and alkali. It is assumed, therefore, that the active principle from *B. subtilis* is a derivative of an alkyldiamine or polyalkylamine.

Because the amount of the factor which we have purified so far from *B. subtilis* cells is very small, it is difficult to determine the chemical structure of the factor.

It is well known that alkyldiamines and polyalkylamines exist in bacterial cells[12,16,17] and bacteriophage[18].

FRASSA showed the protective effect of spermidine on the infectious factor of phage which is essential in the phage infection of protoplasts[19]. ROSANSKY reported the inhibition of the growth of various bacteria by spermine[3]. Recently MAGER reported the protective effect of spermine on bacterial protoplasts in making them more resistant to osmotic shock[20].

On the mechanism of the biological functions of these amines, however, nothing is known. In this respect, the facts that amine-like material from *B. subtilis* and some natural amines stimulate the exoenzyme formation and metabolism of phosphate compounds of the resting cell of *B. subtilis*, are very interesting.

There are some reports of factors which stimulate protein synthesis and nucleic acid synthesis simultaneously.

GALE AND FOLKES reported that glycine incorporation factor purified from *Staphylococcus aureus* stimulated both the incorporation of glycine into protein and

the incorporation of adenine into RNA in the disrupted cells of the same strain[21].

STRAUB showed that arginine is necessary for both amylase formation and the turnover of RNA by a soluble system from pancreas[2]. The effect of the factor from *B. subtilis* is most obvious on the polyphosphate metabolism.

Further there should be some relationship between the metabolism of polyphosphate, the turn-over of RNA and the synthesis of exoenzymes. Further studies on the determination of the chemical structure of the factor and the mechanism of its functions are now in progress.

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