CHANGES IN THE MEMBRANE BOUND ALKALINE PHOSPHATASE OF GLUCOSE AND LACTATE GROWN VEGETATIVE CELLS OF BACILLUS SUBTILIS SB15

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SUMMARY: Vegetative cells of Bacillus subtilis strain SB15 have membrane bound alkaline phosphatase activity. The enzyme purified from glucose-grown cells has greater electrophoretic mobility in 0.1% SDS-polyacrylamide gel than that from lactate grown cells. The enzymes from glucose and lactate grown cells have apparent molecular weights of 70,000 and 85,000 respectively; their K_m values determined with p-nitrophenyl phosphate as substrate are similar and their pH optima are the same at 9.5. The enzymes from both sources dissociated into two subunits after treatment with 6 M urea. The crude enzyme preparations have broad substrate profiles but after the second Sephadex G-100 chromatography their glucose-6-phosphatase and pyrophosphatase activities were lost; in addition, enzyme from lactate grown cells lost its ATPase activity.

INTRODUCTION: We have recently presented evidence (1) that production of alkaline phosphatase by vegetative cells of Bacillus subtilis is derepressed by glucose and glycerol and repressed by lactate, pyruvate and succinate; and that the glucose derepression is reversed by lactate. There are several significant differences in alkaline phosphatase production between the glucose and lactate grown cells, i.e., (i) longer lag in enzyme synthesis and cessation of enzyme formation earlier in the growth phase in lactate grown cells; (ii) higher alkaline phosphatase content of glucose grown cells; (iii) different substrate profiles in glucose and lactate grown cells. In this communication we are presenting evidence that membrane bound alkaline phosphatases of different electrophoretic mobility and substrate specificity, are formed when the carbon source of the growth

medium is changed from glucose to lactate.

METHODS: Organism and culture conditions: The cultures of Bacillus subtilis SB15 were grown in a 40 liter fermenter with phosphate-free peptone medium (2) for 13 hr at 30° C. Protoplasts were prepared according to a procedure published earlier (3) with the substitution of TAC buffer for Tris-HCl buffer. For lysis protoplasts were suspended in protoplasting buffer without any osmotic support.

Phosphatase assay: The hydrolysis of p-nitrophenyl phosphate was measured as described earlier (1); the only exception is the use of 1 M Tris-HCl buffer containing 2 mM $MgCl_2$ (4). Protein was estimated by Lowry's method (5). Units of enzyme activity are expressed as μmoles of p-nitrophenol (PNP) released/min at 37° C. Specific activity is expressed as units/mg of protein.

Column chromatography: DEAE-Sephadex columns (4 x 50 cm) and Sephadex G-100 columns (2.5 x 80 cm) were washed and equilibrated with 0.01 M Tris-acetate buffer, pH 7.4 containing 0.05 M cobalt chloride (TAC), and 0.2 M Mg-acetate. Crude enzyme preparations in TAC buffer containing 0.2 M Mg-acetate were added on the columns and eluted with the same solution. Effluents from the columns were collected in 2 ml volumes at 2 - 4° C. The void volume of the Sephadex G-100 column was determined by blue dextran.

Gel electrophoresis: Electrophoresis in 0.1% SDS-polyacrylamide gel was done at room temperature in 0.1 M TAC buffer at pH 7.4 (6, 7). The samples, mixed with glycerol (10%), SDS (0.1%) and bromophenol blue as a marker, were layered on the gel and electrophoresed at 8 mA/gel for 4 - 5 hrs. After electrophoresis the proteins were fixed in 15% TCA for 2 hrs and stained with 0.2% aqueous coomasie blue; the unreacted dye was

removed electrophoretically with 7.5% acetic acid for 1 hr. For the determination of subunit constitution the enzyme preparations were dialyzed overnight against 0.1 M TAC buffer, pH 7.4, containing 6 M urea. The molecular sizes of the enzymes were determined by comparison to the mobilities of the standard proteins, soya-bean trypsin inhibitor, pepsin, egg albumin, bovine serum albumin, and bovine-γ-globulin.

RESULTS: Alkaline phosphatase as a membrane bound enzyme. Data on the distribution of alkaline phosphatase in different cell fractions is given in Table 1. Though total enzyme activity of glucose grown cells is greater than that of lactate grown cells, the distribution of enzyme in various subcellular fractions is similar. Progressive enrichment of the specific activity in the crude and washed membrane indicates that the membrane is the significant subcellular location for alkaline phosphatase.

The washed membrane fraction contained 85 - 90% of the total cellular enzyme activity. Though repeated washings of the crude membrane in TAC buffer released only 5 - 10% of the enzyme, a high concentration of magnesium acetate readily extracted it. It is known that bacterial membrane devoid of any associated cytoplasmic material represents about 10% of the cell's protein (8); but TAC-washed membrane used in the present experiment contained about 30 - 35% of the total cellular protein. Hence, the enzyme does not appear to be present within the membrane; rather it is firmly associated to it by electrostatic binding. Such binding has also been shown in other strains of B. subtilis (9). Our earlier electron microscopic data also suggested that the sites of enzyme activity are not present within the membrane (2).

Purification of the enzyme: The same procedure was used for purification of the enzyme from cells grown on glucose (G-cells) or on

TABLE 1. SUBCELLULAR DISTRIBUTION OF ALKALINE PHOSPHATASE OF BACILLUS SUBTILIS

	Lactate			Glucose		
	Apase (units)	Protein (mg)	SpAc	Apase (units)	Protein (mg)	SpAc
Whole cell	1.28	1.20	1.06	1.61	1.19	1.35
Protoplast supernatant	0.05	-	-	0.03	-	-
Protoplast	1.35	1.05	1.28	1.80	0.99	1.81
Soluble cytoplasm	0.02	0.29	0.07	0.01	0.31	0.04
Crude membrane	1.23	0.51	2.40	1.62	0.61	2.62
Washings	0.14	0.10	1.40	0.07	0.15	0.47
Washed membrane	1.08	0.38	2.90	1.43	0.41	3.30

Table 1. After lysozyme treatment the supernatant and the intact protoplasts were separated by 15 min centrifugation at 10,000 x g; the protoplasts were lysed and the soluble cytoplasm and crude membrane were separated by centrifugation. The crude membrane was washed three times with TAC buffer and the washings were pooled.

TABLE 2. PERCENTAGE RECOVERY OF THE PROTEIN AND ENZYME ACTIVITY IN DIFFERENT STAGES OF PURIFICATION

Treatment	Lactate			Glucose		
	Enzyme activity	Protein	SpAc	Enzyme activity	Protein	SpAc
B. Cell membrane	100	100	1.6	100	100	2.2
C. IM MgAc extract	38.60	36.2	1.7	41.30	23.50	3.9
D. 0.5 M MgAc extract	13.30	-	-	22.10	3.10	15.8
E. 0.2 M MgAc extract	5.70	1.10	8.1	12.30	2.10	12.6
F. DEAE-Sephadex	5.10	0.21	37.8	9.70	1.11	18.9
G. Sephadex G-100 - 1st	5.06	0.16	50.0	9.50	0.51	41.5
H. Sephadex G-100 - 2nd	1.60	0.06	44.7	5.10	0.21	53.5

lactate (L-cells). The enzymes were extracted from the washed membrane by the procedure described by Takeda and Tsugita (10). The crude enzyme preparations in 0.2 M magnesium acetate were further purified by chromatography once in DEAE-Sephadex and twice in Sephadex G-100

columns. Recovery and specific activities of the enzymes at various stages of purification are shown in Table 2. The higher yield of enzyme activity from G-cells at every stage of purification is probably due to a higher amount of the enzyme contained in those cells.

Significant differences were noted in DEAE-Sephadex elution profiles of the two enzymes. Enzyme from L-cells showed two protein peaks, one of which had the enzyme activity. In contrast, enzyme from G-cells showed a single peak of protein and enzyme activity. The material from L-cells lost at this stage of purification about 80% of the non-alkaline phosphatase proteins with a five-fold increase of specific activity, but a much smaller change in the specific activity was noted for the enzyme from G-cells. The elution profiles of the two enzymes on Sephadex G-100 differ only slightly (Fig. 1). The activity peak of the enzyme from G-cells has a Ve/Vo value of 1.35, compared to 1.31 for the enzyme from L-cells. In both cases one non-alkaline phosphatase protein peak was separated in the second Sephadex G-100 column (Fig. 1B).

Gel electrophoresis: The purity of the enzymes was monitored by 0.1% SDS polyacrylamide gel electrophoresis. It is evident from Fig. 2 that the gel profiles (E) are similar for the crude enzymes from both G- and L-cells. After DEAE-Sephadex chromatography (F), however, the gel patterns differ, showing a greater number of bands for G-cell enzyme than for the enzyme from L-cells. The two enzymes (H) differ quite measurably in electrophoretic mobility, a fact which becomes apparent after partial purification (F, G). The apparent molecular weights calculated from the electrophorograms are 70,000 for the enzyme from G-cells and 85,000 for the enzyme from L-cells. Dialysis against 6 M urea prior to SDS electrophoresis in both cases resolved the single band of the enzymes into

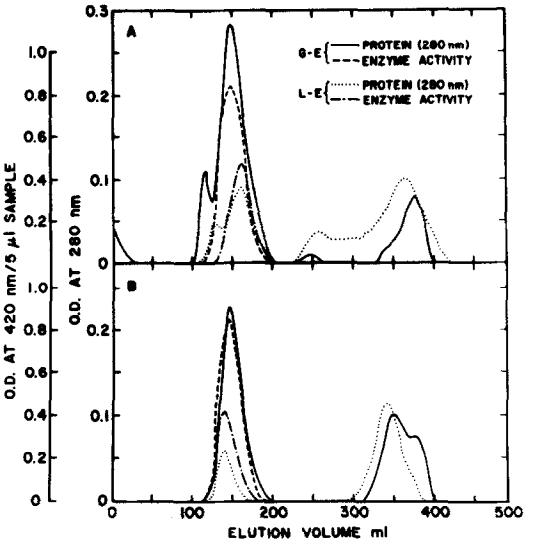

Fig. 1. Chromatography of pooled enzyme activity fractions obtained from DEAE Sephadex column, in Sephadex G-100 column; 0.2 M magnesium acetate in TAC buffer was used for eluting the materials from the column. A: First run; B: second run.

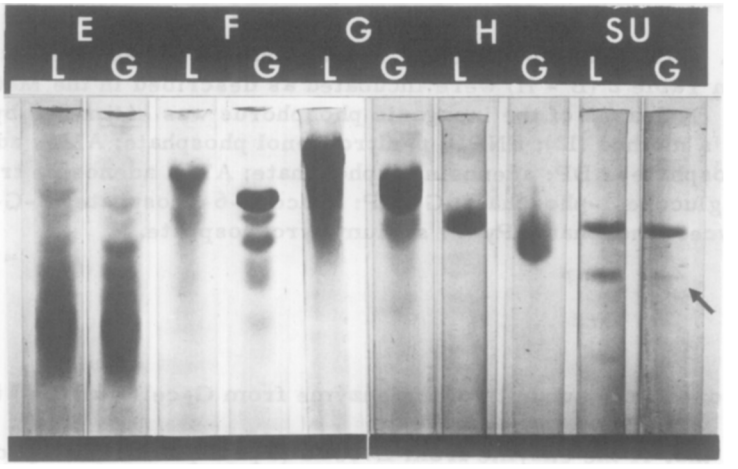

Fig. 2. The profiles of the enzyme at different stages of purification after 0.1% SDS polyacrylamide-gel electrophoresis. L, lactate enzyme; G-glucose enzyme; E-H, same as in Table 2; SU- subunit after 6 M urea treatment.

two clearly defined protein bands (SU) which suggests the presence of two tightly-bound subunits of the enzyme. It should be noted that the relative

TABLE 3. ALKALINE PHOSPHATASE ACTIVITY ON VARIOUS SUBSTRATES RELATIVE TO p-NITROPHENYL PHOSPHATE

	Substrate	A - Whole cell lysate	B	C	D	E	F	G	H
Lactate cell	PNPP	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
	AMP	1.01	0.81	1.08	0.88	0.75	1.39	0.81	0.83
	ADP	1.09	1.18	0.97	0.72	0.70	1.06	0.80	0.68
	ATP	0.66	0.60	0.65	0.47	0.52	0.90	0.53	0.05
	G-1-P	0.41	0.37	-	0.30	0.36	0.84	0.57	0.29
	G-6-P	0.29	0.35	0.38	0.24	0.23	-	0.23	0
	β-G-P	0.73	0.70	1.02	0.78	0.58	1.06	0.81	0.57
	Py-P	5.23	1.20	5.47	0.25	0.17	0.39	0.21	0
Glucose cell	PNPP	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
	AMP	0.87	1.08	0.93	0.88	0.79	0.81	0.70	0.87
	ADP	0.83	1.55	0.92	0.77	0.64	0.74	0.92	0.76
	ATP	0.58	-	0.95	0.53	0.53	0.40	0.42	0.36
	G-1-P	0.64	0.58	-	0.41	0.42	0.41	0.40	0.40
	G-6-P	0.25	0.63	0.40	0.19	0.24	0.39	0.42	0
	β-G-P	0.68	1.13	0.70	0.90	0.57	0.60	0.82	0.45
	Py-P	1.30	1.76	0.95	0.26	0.09	0.19	0.24	0

Table 3. Different substrates (0.5 mM) and enzyme sources as shown in Table 2 (B - H) were incubated as described in the Methods section. Releases of the inorganic phosphorus was estimated by Ernster's method (12); PNPP: p-nitrophenol phosphate; AMP: adenosine monophosphate; ADP: adenosine diphosphate; ATP: adenosine triphosphate; G-1-P: glucose-1-phosphate; G-6-P: glucose-6-phosphate; B-G-P: beta-glycerophosphate; Py-P: sodium pyrophosphate.

density of one of the subunits of the enzyme from G-cells (arrow) is much lower than that of the enzyme from L-cells (equal quantities of proteins were applied to the gels).

Substrates cleaved by the enzyme preparations at various stages of purification: Alkaline phosphatase activities of the whole cell lysate, crude membrane material and enzyme fractions obtained at different stages of purification were compared in the presence of a variety of substrates. The

results are expressed as activity toward substrates relative to that on p-nitrophenyl phosphate (Table 3). It is obvious that except for the very strong cytoplasmic pyrophosphatase activity of L-cells, all other activities are solely membrane bound. Purified enzymes from both types of cells lose their inorganic pyrophosphatase and glucose-6-phosphatase activity. More worthy of note, however, is that the enzyme from L-cells shows almost total loss of ATPase activity whereas this activity was retained by the enzyme from G-cells.

It can be argued that multiple alkaline phosphatases exist in the cell as a loosely-associated functional complex that dissociates upon rigorous purification. Earlier electron microscopic data (2) also showed that in each cell there are only a few discrete membrane bound clusters of alkaline phosphatase activity. This observation, along with that described in the present paper, supports the working hypothesis of multi-alkaline phosphatase complexes.

The pH optima of the enzymes from G- and L-cells in 0.2 M glycine-NaOH buffer are at 9.5. The Km values determined with p-nitrophenyl phosphate as substrate in 0.2 M glycine NaOH buffer, pH 9.5, are 16 and 13 μM respectively.

DISCUSSION: Two important points emerge from the results presented in this paper, (1) the cells of B. subtilis, when grown in media containing different carbon sources, form alkaline phosphatase with differing properties; (ii) alkaline phosphatase may be a multienzyme complex.

The enzymes obtained from glucose and lactate grown cells are similar in that both can be purified by the same procedure and have the same Km and pH optima, yet they differ in electrophoretic mobility and their capability to cleave different substrates. It can be assumed that such changes

in the properties of the enzymes are related to the metabolism of the carbon sources. It is known that alterations of the molecular forms of many enzymes cause specific changes in the related metabolic pathways (11).

The possibility of the existence of a multi-alkaline phosphatase complex is suggested from the observation that, although the crude preparations acted on a variety of phosphate esters, the activities towards some of these substrates were lost from the purified material. It is intriguing that the crude material retained the multiple substrate specificity through several stages of purification; only at the final stage were the activities on some substrates lost. This suggests a fairly tenacious binding of the various components of the complex. Previously we reported (2) that in the repressed cells the alkaline phosphatase activity toward some substrates is more intensively repressed than others, but in both repressed and derepressed cells, there were only a few discrete clusters of membrane bound enzyme activity. To explain these results it was postulated that the cell contained complexes of multiple alkaline phosphatases and individual components of these complexes were repressed to a variable extent. Preliminary biochemical data, presented here, also support this multiple alkaline phosphatase concept. Experiments are in progress on the purification and characterization of the column fractions having no activity on p-nitrophenyl phosphate. Attempts are being made to reconstitute the full substrate specificity of the purified enzyme by reassociating it with different fractions obtained from the column. There may be other interpretations to explain these results, but we offer this as our working hypothesis.

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