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Isolation of a Specific ATP: D-Fructose 6-Phosphotransferase from *Mycobacterium phlei*

Wyodrębnienie specyficznej 6-fosfotransferazy ATP: D-fruktoza z *Mycobacterium phlei*

Изолирование специфической 6-фосфотрансферазы АТР: Д-фруктоза из *Mycobacterium phlei*

Saprophytic mycobacteria can grow on fructose as well as glucose, let the corresponding sugar kinases considerably differ with respect to their activities. Thus, the kinase responsible for glucose phosphorylation is a constitutive enzyme and utilizes inorganic polyphosphate more readily than ATP while that for fructose is formed in response to an inducer and requires ATP as the principal phosphate donor (10, 11).

Previous results suggested that the phosphorylation reactions involving inorganic polyphosphates might be due to enzymes specifically different from those which use ATP (9). Recent work showed, however, that pure polyphosphate-glucose phosphotransferase of *Mycobacterium tuberculosis* $H_{37}Ra$ was capable of utilizing both phosphate donors (12). On the other hand, in agreement with the former suggestion a separate ATP-depedent hexokinase (5) has been found in mycobacteria.

The aim of this study has been to re-examine the fructokinase activity of *Mycobacterium phlei* with the use of some hitherto unemployed methods.

MATERIALS AND METHODS

Chemicals. Sources of reagents and substrates were as previously described (10—12).

Microorganisms and cultivation. *Mycobacterium phlei* was used throughout. In preliminary experiments, some other strains were tested i.e., *M. friburgi, M. hauduroy, M. jucho, M. smegmatis, Mycobacterium* sp. 279 and *Mycobacterium* sp. 607. All the strains were maintained in this laboratory for several years by serial mass transfers on Lowenstein slants. They were grown on fructose--glutamate-citrate-salts media (initial *pH* 6.6) as reported earlier (10, 11).

Preparation of cell-free extract. Washed mycobacteria were disrupted by sonication in an MSE disintegrator (100 W, 20 kc/s) using 20% cell suspension in 0.04 M Tris-HCl buffer (*pH* 7.6) containing 1 mM β-mercaptoethanol, 1 mM EDTA and 50 mM fructose (further referred to as T-ME-EDTA-F buffer). About 30 ml volumes were sonicated for 2×5 min (under cooling) with subsequent centrifugation at 18 000×g.

Protein determination. The protein content in extracts and in partially purified enzyme solutions were determined by the method of Lowry et al. (6) using bovine serum albumin as a standard.

Enzyme assays. Fructokinase activity was measured on the basis of either fructose or ATP utilization. For the assays, a stock mixture was prepared which contained: 120 mM Tris-HCl buffer (*pH* 8.0), 10 mM ATP, 7.5 mM $MgCl_2$, 10 mM fructose and 250 mM KCl. If not otherwise specified, a 0.40 ml volume of the stock mixture was incubated at 37°C with 0.1—0.2 ml enzyme. At the beginning and at desired time intervals (usually 10—20 min) aliquots of the incubation mixture were withdrawn and either substrate was determined. In the case of crude extract, 0.2 ml volumes of the incubation mixture were transferred to centrifugal test tubes containing 2.0 ml H_2O with subsequent additions o 5% $ZnSO_4$ (0.3 ml) plus 0.3 N $Ba(OH)_2$ (0.5 ml). After stirring with a glass rod, the precipitate was spun down and in the supernatant (2.0 ml) unutilized fructose was measured by the Roe method (13). In the case of partially purified enzyme preparations, 0.1 ml volumes of the incubation mixture were transferred to test tubes containing 2.9 ml H_2O plus 1.0 ml 4 N H_2SO_4 and heated for 10 min at 100°C (boiling water-bath). After cooling, orthophosphate was measured by the Fiske-Subbarow method (13). Color readings were taken in a spectrocolorimeter Spekol at 500 and 660 nm, respectively.

Inorganic polyphosphate-glucose phosphotransferase (EC 2.7.1.63) was determined as described earlier (12).

One unit of enzyme activity is defined as 1 μmol of substrate utilized per minute under the assay conditions. Specific activity is expressed in units per mg protein.

Chromatography. Ion-exchange chromatography was carried out using Whatman DEAE-cellulose DE 52 equilibrated with T-ME-EDTA-F buffer according to the instructions of the producer. For elution, both stepwise and linear gradients of KCl were used.

Gel filtration. Glucose and fructose kinase activities were separated on Sephadex G-100 gel. Enzyme solutions were freed from ammonium sulfate and/or fructose by filtration on either Sephadex G-100 or Sephadex G-25 columns. For both equilibration and elution, T-ME-EDTA buffer with or without fructose was employed.

The molecular weight of fructokinase was estimated by gel filtration on Sephadex G-100 according to the method of Andrews (1).

Ionophoresis. Sugars and phosphate esters were analysed by paper ionophoresis which was run at room temperature at 600 V using Whatman filter paper no. 3 (42×24 cm) and pyridine-acetic acid-water (15:50:2460, v/v) as a buffer (2). Reducing spots were visualized with $AgNO_3$-NaOH reagent.

Polyacrylamide gel electrophoresis. The method of Davis (3) was employed but with spacer gel omitted. Appropriate enzyme samples in 10% saccharose were electrophoresed using 5×0.6 cm gels (7% acrylamide), Tris-glycine buffer (*pH* 8.6) and constant electric current of 2—3 mA/tube. Electrophoresis was run at 4°C for about 2 h with bromothymol blue as a marker. Fructokinase actvity was detected with a system which contained: 1.35 ml of 0.2 M Tris-HCl buffer (*pH* 8), 0.1 ml of 0.1 M $MgCl_2$, 20 mg agar (dissolved in boiling water-bath), 0.15 ml of 0.1 M fructose, 0.1 ml of 0.1 M ATP, 0.1 ml of nitrotetrazolium blue (0.8 mg), 0.02 ml of phenazine methosulfate (0.08 mg), 0.1 ml of phosphohexoisomerase (about 0.2 unit — a preparation obtained by Dr. O. Szymona), 0.15 ml of 0.01 M NADP and 0.1 ml of G-6-P dehydrogenase (hexokinase-free, dialysed). After mixing by inversion, an electropherogram was introduced to the above system and color development observed for 30—60 min.

RESULTS

In preliminary experiments various *Mycobacterium* strains were examined for their fructokinase activity. The organisms were grown on fructose-containing media for several days, then collected, washed with distilled water and disintegrated as in Methods. The resulting extracts served as an enzyme source.

Table 1 shows fructokinase activity as determined with the use of ATP. It is evident that the extracts tested were capable of phosphorylating fructose to a variable extent, *M. phlei* and *M. hauduroy* being most active. Attempts to detect the fructokinase activity with the use of inorganic polyphoshpates gave negative results.

Table 1. Fructokinase activity in extracts from various *Mycobacterium* strains grown on fructose-containing media

Strain	Age of culture (days)	Wet wt (g/100 ml)	Final *pH* of culture fluid	Spec. activity* (miliunits/ /mg protein)
M. friburgi	5	4.4	7.6	5
M. hauduroy	5	3.6	7.5	14
M. jucho	4	5.2	7.6	5
M. phlei	6	5.6	7.6	22**
M. smegmatis	4	5.7	7.7	9
M. sp. 279	4	6.0		4
M. sp. 607	4	4.5	7.5	7

* Activity was determined on the basis of decrease in fructose concentration.
** Average value for three independent cultures.

ISOLATION OF FRUCTOKINASE FROM *M. phlei*

Further experiments aimed at obtaining ATP-dependent fructokinase in a separate state. To this end, *Mycobacterium phlei* was employed. The first step consisted in preparing cell-free extract from about 50 grams of wet weight bacteria. The extract in a volume of 200 ml was then chromatographed on a DEAE-cellulose column (3×3.5 cm) which had been equilibrated with T-ME-EDTA-F buffer (*pH* 7.6). After adsorption, the column was washed with the same buffer and subsequently eluted with KCl. Fractions of 6 ml were collected at a flow rate of 60 ml/h, and both protein content and kinase activities determined.

Figure 1 shows that fructokinase activity emerged from the column upon addition of 0.1 M KCl. At the same time, a large part of polyphosphate-glucose phosphotransferase was eluted. This enzyme proved to occur in mycobacteria in two molecular forms one of which passes through the column unretarded while the other from becomes adsorbed (7). In order to separate fructokinase from this latter form, the most active fractions (45—54) were combined and concentrated with ammonium sulfate at 75% saturation. During treatment with ammonium sulfate, the preparation was adjusted to *pH* 7 by additions of 0.4 M Tris. After centrifugation, the precipitate was dissolved in 4 ml of T-M-EDTA-F buffer, and applied to a Sephadex G-100 column (2.6×87 cm) which had been equilibrated with the same buffer. The filtration was conducted at a flow rate of 15 ml/h. Figure 2 shows the elution profile indicating two distinct peaks of phosphotransferase activities towards glucose and fructose, respectively. The most active fractions containing fructokinase (42—54) were pooled and rechromatographed on DEAE-cellulose. In this case, a higher column (1.5×15 cm) and a linear KCl concentration gradient (0/0.4 M, pH 7.6) was used. Fractions of 6 ml were collected at a flow rate of 20 ml/h. As one can see in Fig. 3, fructokinase became eluted as a symmetrical peak upon influence of 0.10—0.14 M KCl. The results of the fractionation procedure are summarized in Table 2. It is clear that the adopted method gave an approx. 40-fold purification with about 24% recovery.

The final enzyme solution, when tested by polyacrylamide gel electrophoresis revealed one single activity band with fructose and ATP as the subtrates. Its *R* value relative to the marker was about 0.52. There was no activity with polyphosphate as the phosphate donor, and no reaction with glucose. Control gels incubated without fructose or without ATP gave no reaction either.

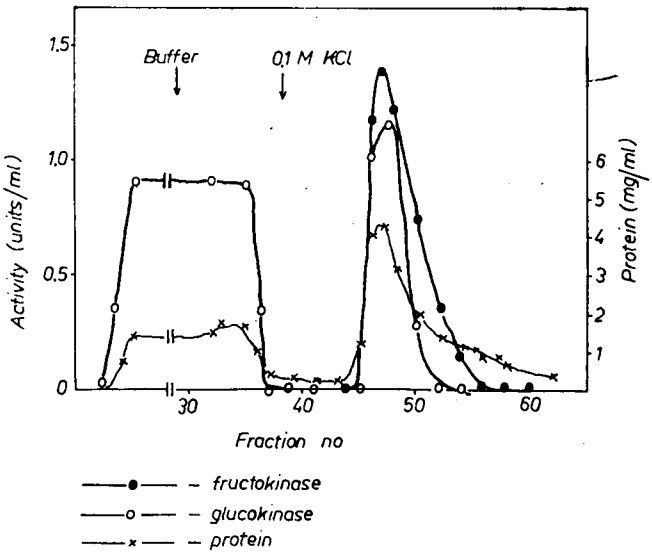

Fig. 1. DEAE-cellulose chromatography of cell-free extract from *M. phlei*. Elution with buffer and KCl as indicated

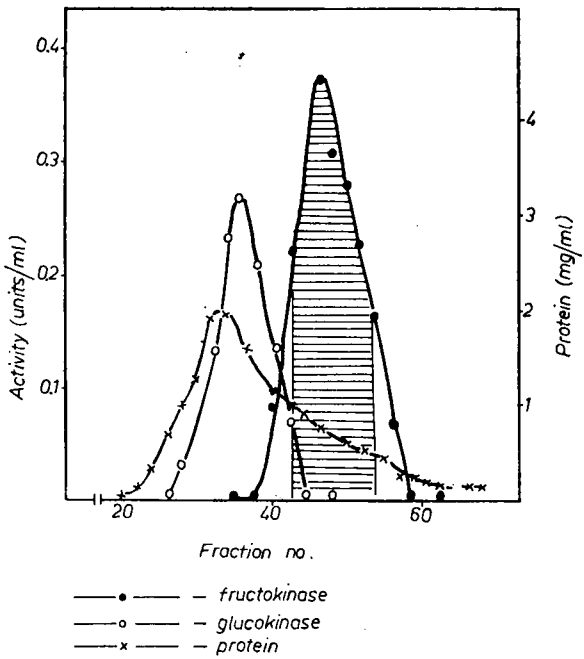

Fig. 2. Filtration of $(NH_4)_2SO_4$-precipitated material on Sephadex G-100. Striated area indicates the most active fractions pooled for further treatment

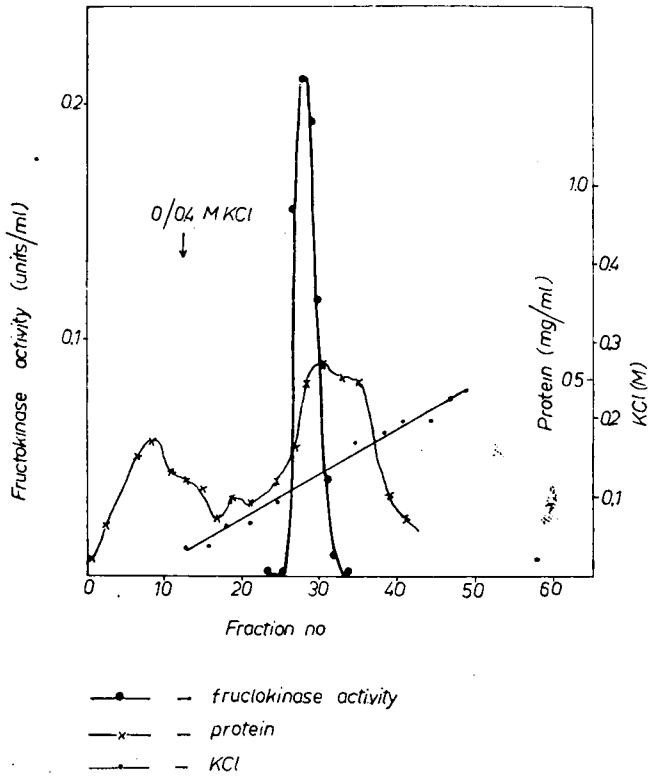

Fig. 3. DEAE-cellulose chromatography of Sephadex G-100 eluate. Elution with a linear KCl concentration gradient

Table 2. Purification of fructokinase from *M. phlei*

Fraction	Vol. (ml)	Total protein (mg)	Activity (units)		Purification factor	Yield (%)
			total	per mg protein		
(I) Extract	200	1200	36.0	0.03		100
(II) DEAE-cellulose (stepwise)	47	196	35.3	0.18	6	96
(III) Sephadex G-100	70	70	21.9	0.32	10	60
IV DEAE-cellulose (linear gradient)	22	7.7	8.8	1.14	38	24

PROPERTIES OF FRUCTOKINASE FROM *M. phlei*

Figure 4 shows that the *pH* optimum activity is comprised between 7.5 and about 8.5 which agrees with former data (10).

The effect of the fructose concentration is represented in Fig. 5. Although the method used gave somewhat scattered results it is evident that the enzyme became saturated at about 8 mM fructose.

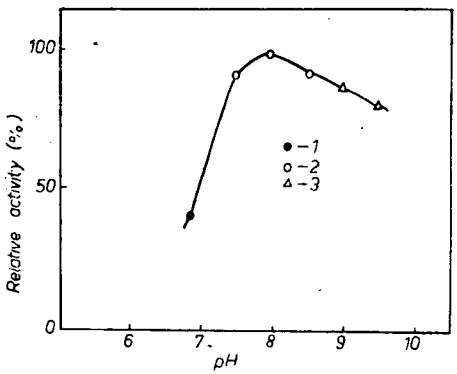

Fig. 4. Effect of *pH* on fructokinase activity. Standard assay conditions except for buffer as indicated 1 — Tris-maleic acid, 2 — Tris-HCl, 3 — glycine-NaOH. DEAE-cellulose eluate (step IV) was used as enzyme source

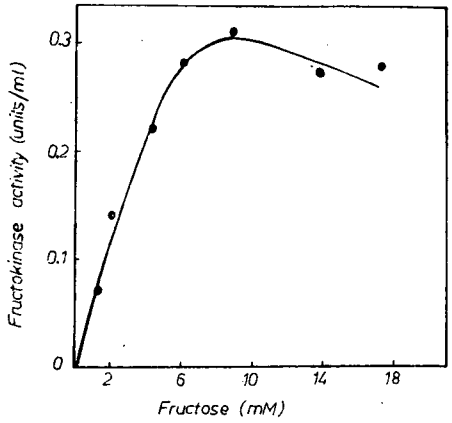

Fig. 5. Effect of D-fructose concentration on the reaction rate. Standard assay conditions except varying fructose as indicated. DEAE-cellulose eluate (step IV), after removal of fructose by Sephadex G-25 filtration, was used as enzyme source

The effect of temperature was studied using the enzyme with and without fructose added. In a series of assays, aliquots of either enzyme were heated for one minute (water bath) at various temperatures, and after cooling activity was determined. Figure 6A indicates rapid inactivation upon heating above 50°C irrespective of whether fructose was present or not. When the enzyme (Sephadex G-100 eluate) was stored at 4°C, a gradual decrease in activity was observed, 50% being left after 9 and 6 days in the presence and absence of fructose, respectively (Fig. 6B).

Specificity of the enzyme was studied by incubating appropriate reaction mixtures with different sugars followed by filter paper ionophoresis. Figure 7 demonstrates that the enzyme was active with fructose but inactive with glucose, glucosamine, mannose and tagatose. Attempts to replace ATP by inorganic polyphosphates ended in failure (not shown).

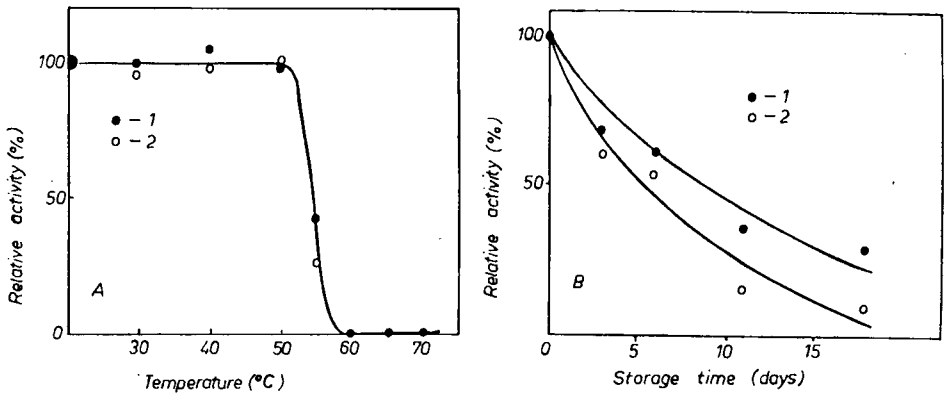

Fig. 6. Stability of fructokinase in the presence (1) and absence (2) of D-fructose. A — the step IV enzyme preparation was freed from fructose by Sephadex G-25 filtration, then 0.4 ml samples were heated for 1 minute at indicated temperatures without or with 50 mM fructose added. B — Sephadex G-100 eluate was stored at 4°C for indicated times without or with 50 mM fructose added. Activity was assayed under standard conditions as in Methods

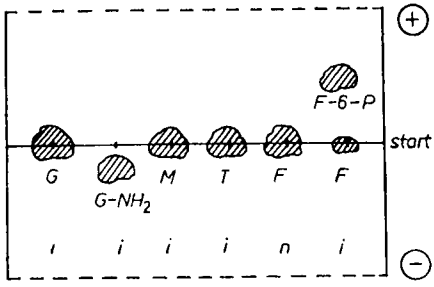

Fig. 7. Paper ionopherogram of the incubation mixtures with indicated sugars: glucose (G), glucosamine (G-NH_2), mannose (M), tagatose (T) and fructose (F). The mixtures contained in a final volume of 0.60 ml: 3 µmol of sugar, 4 µmol of ATP, 3 µmol of $MgCl_2$, 100 µmol of KCl, 50 µmol of Tris-HCl buffer (*pH* 8.0) and 0.2 ml of enzyme (Sephadex G-100 eluate, fructose-free). Control samples (*n*) were deproteinized with 0.05 ml of 60% $HClO_4$ immediately, incubated samples (i) — after 1 hour of incubation at 37°C. The mixtures were then neutralized with solid $KHCO_3$, and 0.04 ml portions of the supernatants used for analysis. Further details are given in Methods

The molecular weight of fructokinase was determined by the Andrews method (1). To this end, a Sephadex G-100 column (1.5×90 cm) was equilibrated with 0.02 M phosphate buffer (*pH* 7.2) and 1.5 ml of enzyme (DEAE-cellulose eluate) passed through the gel. To compare with, following marker proteins were used in separate runs: myoglobin (17 800), trypsin (24 000), hemoglobin (64 500) and aldolase (149 000). The void volume (V_0) was established with Dextran blue. As it can be seen (Fig. 8), the molecular weight of *M. phlei* fructokinase amounts to 54 300 daltons.

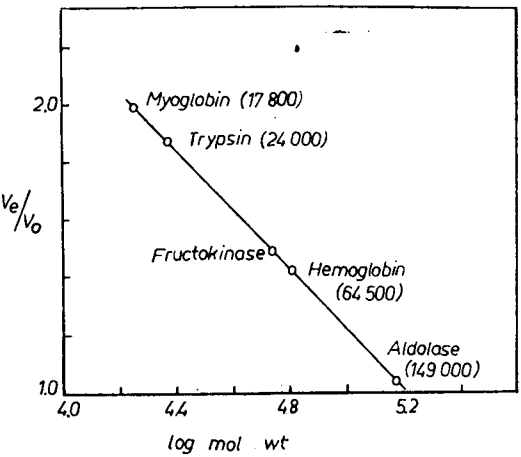

Fig. 8. Estimation of the molecular weight of fructokinase. DEAE-cellulose eluate (1.5 ml) was passed through a Sephadex G-100 column (1.5×90 cm) which had been equilibrated with myoglobin, trypsin, hemoglobin and aldolase. Elution was carried out at a flow rate of 12 ml/h. Fractions of 3 ml were collected and fructokinase activity measured as in Methods

DISCUSSION

The ability of saprophytic mycobacteria to grow at the expense of fructose suggested the existence of a kinase phosphorylating this sugar. Indeed, previous work brought evidence for this reaction to occur in *Mycobacterium phlei* (10, 11). When fructose-grown cells were disintegrated by grinding with alumina or corundum, an extract capable of phosphorylating fructose with ATP, and eventually with inorganic polyphosphates was obtained. Fractionation of the extract with ammonium sulfate and Sephadex G-100 made it possible to separate an ATP-dependent fructokinase from polyphosphate-dependent glucokinase but not from polyphosphate-dependent fructokinase. This latter activity detectable in eluates from Sephadex G-100 columns entirely disappeared during subsequent chromatography on DEAE-cellulose. The above result was taken as evidence for nonidentity of the enzymes phosphorylating fructose with the two phosphate donors (10).

The results of the present work confirm the occurrence of the fructokinase as a separate enzyme. Using a different method of disintegration (ultrasounds) we obtained extract which phosphorylated fructose with ATP but not with polyphosphates. When crude extract was directly chromatographed on DEAE-cellulose at *pH* 7.6, the fructokinase activity was separated from the heavy form of polyphosphate-glucose phosphotransferase but not from the "normal" 110 000 mol. wt enzyme (7, 12). The latter form could be removed, however, by Sephadex G-100 filtration. In fact, the fructokinase revealed a two-fold lower molecular weight (54 000) and so emerged later.

The partially purified fructokinase was most active at *pH* 7.5—8.6 and underwent inactivation when heated above 50°C. In contrast to mycobacterial polyphosphate-glucose phosphotransferase which required glucose as a protective agent, the stability of *M. phlei* fructokinase was markedly less dependent on fructose.

It proved to be specific for fructose and thus should be classified as ATP: D-fructose 6-phosphotransferase (EC 2.7.1.4).

When examined on polyacrylamide gel electrophoresis, one activity band with a R_m value of 0.52 could be stated.

Unfortunately, we were not able to find polyphosphate-fructose phosphotransferase. Some attempts to detect such an activity at various purification stages gave negative or erratic results.

Recently, Indian authors reported on ATP-dependent fructokinase in *Mycobacterium smegmatis*. They searched for the enzyme in a study concerning transport of fructose into cells (4).

A highly specific fructokinase was found by Sabater et al. in *Streptomyces violaceoruber* (8). The enzyme was inducible, like *M. phlei* fructokinase, and revealed complex kinetic properties. It showed a lengthy plateau of optimal *pH* in the alkaline range from about 8 to 10.

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STRESZCZENIE

Wyhodowane na podłożu z fruktozą komórki *Mycobacterium phlei*, dezintegrowano w aparacie ultradźwiękowym MSE i z ekstraktu wyodrębniano 6-fosfotransferazę ATP : D-fruktoza (fruktokinazę), stosując kolejno: 1) wysalanie siarczanem amonu, 2) chromatografię na DEAE-celulozie, 3) sączenie na sefadeksie G-100 i 4) rechromatografię na DEAE-celulozie.

Końcowy, ok. 40-krotnie oczyszczony, enzym okazał się swoisty dla wyżej wymienionych substratów (nie zużytkowywał polifosforanów i nie fosforylował innych heksoz) i dawał w żelu poliakrylamidowym jedną strefę aktywności. Optimum *pH* reakcji fosforylacyjnej wynosiło 7,5—8,5, nasycające enzym stężenie fruktozy — ok. 8 mM. Ogrzewanie powyżej 50°C powodowało szybką denaturację. Ciężar cząsteczkowy, oznaczony metodą filtracji żelowej, wynosił ok. 54 000 daltonów.

Aktywność fruktokinazową stwierdzono również w ekstraktach szeregu innych saprofitycznych szczepów *Mycobacterium*.

РЕЗЮМЕ

Выращенные на фруктозовой среде клетки *Mycobacterium phlei* разрушали в ультразвуковом аппарате MSE и из полученного экстракта изолировали 6-фосфотрансферазу АТР : Д-фруктоза (фруктокиназа), применяя следовательно: 1) высаливание $(NH_4)_2SO_4$; 2) хроматографию на ДЭАЭ-целлюлозе; 3) фильтрацию на сефадексе G-100 и 4) рехроматографию на ДЭАЭ-целлюлозе.

Финальный, 40-кратно очищенный фермент был специфический для вышеупомянутых субстратов и проявлял в полиакрилоамидном геле одну зону активности. Оптимум *pH* фосфорилирования — 7,5—8,5, насыщающая концентрация фруктозы — 8 mM. Нагревание выше 50°C быстро инактивирует фермент. Молекулярный вес обозначенный методом гель-фильтрации составляет 54 300.

Фруктокиназовую активность обнаружено также в экстрактах из ряда других сапрофитов *Mycobacterium*.