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Isolation of Soluble Preparation of Phosphoproteins from *Escherichia coli*

Izolacja rozpuszczalnego preparatu fosfobiałek z *Escherichia coli*

Изоляция растворимого препарата фосфобелков из *Escherichia coli*

Phosphoproteins (P-proteins) are the class of acidic proteins containing phosphate esterified with serine or threonine residues. In some cases phosphate can be linked to -NH- group of imidazole ring of histidine (8).

Phosphoproteins have been isolated from a number of animal tissues as well as from some bacterial species. There are many experimental data suggesting the possible biological functions of mammalian phosphoproteins in activation of genetic material, oxidative phosphorylation etc. Our knowledge about bacterial phosphoproteins, their localization inside the cell and their function is still obscure. The highest concentration of P-proteins is in the logarithmic phase of growth of bacterial culture and it significantly decreases thereafter to approximately 50% of a value in the stationary phase.

These observations might suggest that P-proteins play a specific role in an intensively metabolizing cell and are not the only stored material.

The aim of this paper was to isolate ester linked phosphoproteins from *E. coli* for chemical characterization. A preliminary account has been reported (6).

MATERIAL AND METHODS

Bacterial culture: *Escherichia coli* strain K12 (Hfr 82 met−) was used for all experiments. Cells were grown in Davis medium supplemented with

25 μg/ml of methionine under aerated conditions at 37°. Bacteria were harvested in logarithmic phase of growth by low speed centrifugation and washed twice with 0.05 M Tris-HCl buffer, *pH* 7.2.

Bacterial extracts: Cell-free extracts were prepared by mechanical disruption of bacterial mass with corund as previously described (3).

Isolation of cytoplasmic membranes: Spheroplasts prepared by lysozyme method of Flessel et al. (2), were suspended in redistilled water and after their breakage the membranes were separated from cytoplasm by centrifugation at 20,000 x g and then washed five times with 0.9% NaCl.

Analytical methods: Protein was determined by the method of Lowry et al. (9), and inorganic phosphate according to Hürst (5) after combustion of samples in the presence of 10 N H_2SO_4 and 2 N HNO_3 at 160° during 1 hr.

Reagents: TEAE-cellulose, Tris (hydroxymethylaminomethan) were the products of Serva, West Germany. Folin reagent, mercaptoethanol, and lysozyme were purchased from Sigma Chemical Company, USA. DNA-se was from Worthington Biochemical Corporation, USA and methionine from British Drug Houses All other reagents were the products of P.O.Ch. Gliwice.

RESULTS

A modified method of Winder and Donney (12) for isolation of P-proteins was employed (scheme 1). 1 g of acetone powder of bacteria (vacuum dried) was used for the extraction procedures. The final insoluble residue amounted to approximately 80% of starting material and contained 0.32 μg Pph/mg protein.* Such a material subjected to hydrolysis followed by paper chromatography (see below) appeared to contain phosphoserine as a constituent of phosphoproteins.

Solubilization of phosphoproteins

In order to obtain a soluble phosphoprotein preparation a slightly modified method of Pinna and Wadkins (10) was used. 500 mg of final preparation collected after extraction procedure were suspended in 75 ml of solution containing 8 M urea, 1.0 M LiCl, 0.005 M mercaptoethanol and 0.05 M Tris-HCl, *pH* 7.2. The suspension was allowed to stand at room temperature for 6 hrs, then was dialyzed against 40 volumes of 0.05 M Tris-HCl, *pH* 7.2, at 4° during 48 hrs.

Insoluble material was removed by centrifugation and the supernatant was acidified with 1.0 M CH_3COOH to *pH* 4.8. This procedure allowed for almost complete precipitation of soluble P-proteins. The precipitate was dissolved in 5 ml of 0.05 M Tris-HCl, *pH* 7.2. The slightly opalescent solution thus obtained contained an approximately 14% P of the extracted sample.

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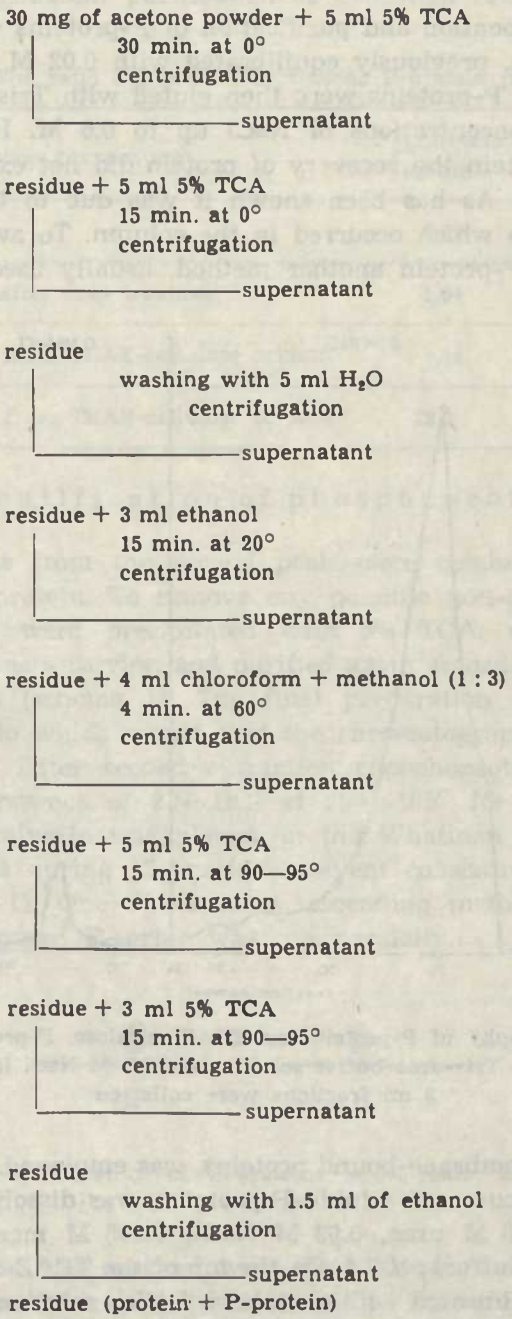
* P_{ph} = phosphoprotein phosphorus.

Scheme 1. Fractionation of *Escherichia coli* acetone powder



TEAE-cellulose chromatography

Further fractionation and purification of P-proteins were carried out on TEAE column, previously equilibrated with 0.02 M NaCl in 0.05 M Tris-HCl, *pH* 7.2. P-proteins were then eluted with Tris buffer containing increasing concentrations of NaCl up to 0.6 M. In spite of good separation of protein the recovery of protein did not exceed 20% of the starting material. As has been shown it was due to the precipitation of phosphoprotein which occurred in the column. To avoid spontaneous precipitation of P-protein another method, usually used for separation

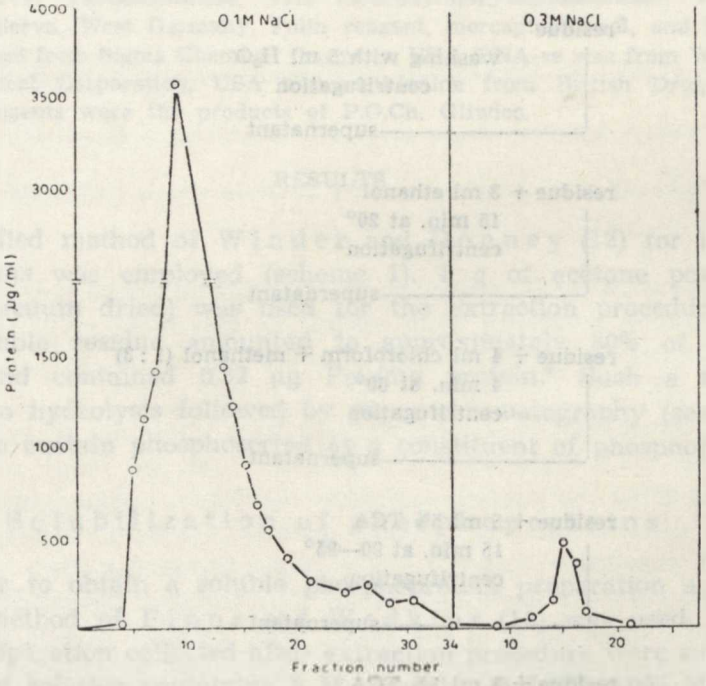


Fig. 1. Chromatography of P-proteins on TEAE-cellulose. P-proteins were eluted with: 0.1 M NaCl in Tris-urea buffer solution and 0.3 M NaCl in the same buffer. 3 ml fractions were collected

of organella or membrane-bound proteins, was employed. The precipitate of *pH* 4.8, as a source of soluble P-proteins, was dissolved in the mixture containing: 8 M urea, 0.02 M NaCl, 0.005 M mercaptoethanol in 0.05 M Tris-HCl buffer, *pH* 7.2. On the top of the TEAE-cellulose column (50×1.5 cm), equilibrated with the above buffer solution, 37 mg protein sample (54 µg P) was placed. Separation of proteins is shown in Fig. 1. This method allowed for good separation of proteins from P-protein

with the yield of approximately 85%. The high ratio of P_{ph} to protein indicates the significant purification of P-protein (Table 1).

Tab. 1. The ratio P_{ph}/protein in various P-protein preparations

P-protein preparation	P_{ph}/protein μg/mg	Degree of purification
Insoluble P-protein	0.32	1
Soluble P-protein after urea treatment	1.64	5
0.1 M NaCl eluate from TEAE-cellulose column	0.54	1.7
0.3 M NaCl eluate from TEAE-cellulose column	22.0	69

Identification of phosphoprotein

The fractions from the second peak were combined and used to identify the P-protein. To remove any possible non-protein impurities phosphoproteins were precipitated with 5% TCA, after addition of bovine albumin as a carrier, and purified again according to the method previously used (scheme 1). The final preparation contained a high P_{ph}-protein ratio which meant that the chromatographed material was relatively pure. After second extraction phosphoproteins were hydrolyzed in the presence of 2 N HCl at 100—105° for 12 hrs. A small aliquot of hydrolysate was placed on the Whatman No. 1 paper and chromatographed during 12 hrs in a solvent consisting of: n-propanol, NH_3, H_2O (7:2:1). One dimensional ascending method was used. As a control a standard P-serine was run paralelly.

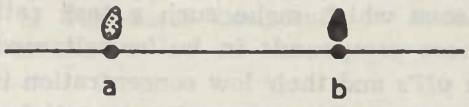


Fig. 2. Paper chromatography of P-proteins hydrolysate; a — hydrolysate, b — P-serine

Chromatogram was sprayed with the solution of Hanes-Isherwood (4). Then it was air-dried, heated at 50° for 3 min. and exposed to UV light for 20 minutes. A small, blue spot corresponding to P-serine was detected in hydrolyzed material (Fig. 2).

Free and membrane-bound phosphoproteins

A low solubility of P-proteins in the absence of urea might suggest that most of them exist in bacterial cell as a membrane-bound protein. To test this there was determined the concentration of phosphoproteins in bacterial extract and in the isolated membrane fraction. As one can see in Table 2 there is a remarkable difference between the content of

Tab. 2. Concentration of protein and phosphoprotein P in various bacterial fractions

Bacterial fraction	Total concentration of protein in mg	Total concentration of phospho-protein P in ug	Ratio P_{ph}/protein µg/mg	Content of P-phospho-protein %
Cell-free extract	122	33	0.27	29
Cytoplasmic membrane fraction	224	79	0.352	71

P-protein in both the examined fractions. Even if one assumes that a disruption of cells by mechanical desintegration was incomplete (80—85% in this method) it seems that at least 60% or more P-protein was bound to the membrane fraction. Furthermore P_{ph}-protein ratio was higher in the membrane preparation than in the cell-free extract.

DISCUSSION

Bacterial phosphoproteins have not yet been isolated and characterized. The main reasons which make such a task rather difficult are: low solubility of these compounds in buffer-salt media, instability in neutral and alkaline *pH's* and their low concentration in a bacterial cell. The methods used in our studies appeared to be satisfactory.

The preparation obtained by TEAE-cellulose chromatography showed a very high P-protein ratio and approximately 70-fold purification. Detection of P-serine and observed almost the same ratio of P_{ph}/protein after second extraction procedure gave additional evidence that the chromatographed sample contained highly purified P-protein preparation. The low yield of the solubilization process can be ascribed to the partial denaturation of material which occurred during extraction

procedure. Nonetheless the application of this method to the isolation and purification of native P-proteins can be useful for further characterization of these compounds.

The experiments confirmed previous suggestion (1,11) that P--proteins in bacterial cells are bound or integrated with cytoplasmic membrane. It could therefore indicate that the function of P-proteins in bacteria is somehow related to some processes in which cytoplasmic membrane can participate.

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STRESZCZENIE

Preparat forsfoproteidowy wyizolowany z komórek *Escherichia coli* oczyszczono 70-krotnie przy pomocy metod ekstrakcji, solublizacji w obecności mocznika i chromatografii na TEAE-celulozie. P-proteid zawierał fosfor związany z seryną. Wykazano, że w komórkach bakteryjnych fosfobiałko występuje głównie we frakcji błon cytoplazmatycznych.

РЕЗЮМЕ

Изолировали 70-кратно очищенный фосфобелковый препарат из клеток *Escherichia coli* при помощи методов экстракции, солюблизации и при наличии мочевины и хроматографии на TEAE-целлюлозе. Р-протеид содержал фосфор, связанный с серином. Обнаружили, что в бактерийных клетках фосфобелок выступает в основном во фракции цитоплазматических мембран.

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