EVIDENCE FOR ADSORBED PROTEOLYTIC ACTIVITY ON ISOLATED YEAST PHOSPHOFRUCTOKINASE

Wolfgang DIEZEL, Karl NISSLER, Werner HEILMANN, Gerhard KOPPERSCHLÄGER and Eberhard HOFMANN

Institute of Physiological Chemistry, Karl-Marx-University, Leipzig, German Democratic Republic

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1. Introduction

Phosphofructokinase may be isolated in two distinguishable molecular forms: one of them sediments with 17 S, the other with 19 to 20 S. The 17 S enzyme is composed of only one kind of subunit having a mol. wt. of 96 000 daltons, whereas the 19 S enzyme consists of two types with mol. wt. of 96 000 and 130 000*, respectively. In the course of storage the 19 S enzyme is converted to the 17 S form. Simultaneously the 130 000 subunit disappears, leaving only the 96 000 subunit together with smaller molecular material as degradation products which may be detected by SDS electrophoresis [1–3].

From these findings it was concluded that isolated yeast phosphofructokinase, which has been found to be homogeneous with analytical ultracentrifugation as well as with polyacrylamide gel electrophoresis undergoes evidently a proteolytic breakdown. This proteolytic activity appears to be strongly attached to the phosphofructokinase protein.

In this paper it will be shown that yeast phosphofructokinase contains indeed a proteolytic contamination which attacks predominantly the 130 000 subunit. The action of this protease on phosphofructokinase is suppressed by F-6-P** and ATP as substrates of the target enzyme as well as by ammonium sulphate.

2. Materials and methods

Yeast phosphofructokinase (EC 2.7.1.11; specific activity 110 to 140) has been purified according to [4] in presence of 1 mM PMSF. The heat treatment of the enzyme has been omitted. The purified product was shown to be homogeneous in the analytical ultracentrifuge as well as in PAA gel electrophoresis. The necessary reagents have been purchased from Serva GmbH, Heidelberg (GFR). ^{14}C-labeled potassium cyanate has been obtained from Isocommerz GmbH, Dresden (GDR).

The analytical PAA gel electrophoresis in presence of 0.1% SDS has been performed according to [5]. The protein bands were stained with Coomassie Brilliant Blue G 250 [6]. The preparative PAA gel electrophoresis in presence of 0.1% SDS has been carried out using equipment developed in our laboratory [7].

The proteolytic activity has been determined according to Roth et al. [8] using ^{14}C-labeled hemoglobin, which has been denatured by 1.0% SDS. Radioactivity was measured with a Tricarb scintillation counter Type 3375. One unit (U) of proteolytic activity is referred to the breakdown of 1 μM hemoglobin per min. Protein was determined according to Heil and Zillig [9].

* The value of the larger subunit depends on the molecular weight of the β-galactosidase subunit chosen for calculation from the calibration curve of SDS-electrophoresis. Here 135 000 daltons for the β-galactosidase subunit were taken [14].

***Abbreviations* used: sodium dodecyl sulphate: SDS; fructose-6-phosphate: F-6-P; phenylmethylsulfonylfluoride: PMSF; polyacrylamide: PAA.

Table 1
Measurement of proteolytic activity in three different preparations of yeast phosphofructokinase.

Preparation	1	2	3
Specific proteolytic activity (μU/mg protein)	63	81	70
Percentage of specific proteolytic activity of the final product to that of the crude extract	6.4%	3.1%	8.3%

3. Results and discussion

3.1. *Demonstration of proteolytic activity in preparations of phosphofructokinase*

Yeast phosphofructokinase which was otherwise shown to be homogeneous contains a proteolytic contamination as revealed by the sensitive radioactive assay procedure (table 1). The following treatments have been found to be ineffective in removing the protease from the phosphofructokinase protein: chromatography on DEAE-cellulose, repeated gel filtration on Sepharose 6 B, sucrose density gradient centrifugation as well as PAA gel electrophoresis. Table 2 demonstrates that after two succeeding passages of the isolated enzyme through Sepharose 6 B the specific protease activity remains rather constant. Hence, the proteolytic contamination appears to be more or less tightly adsorbed to phosphofructokinase. PMSF has only a poor inhibitory effect on this activity.

With affinity chromatography by using denatured protein as protease binding agent covalently fixed to a solid support, a partial separation of both enzymatic activities was obtained. Details will be published in a separate paper together with a new and rapid isolation procedure of yeast phosphofructokinase, with very low proteolytic contamination.

3.2. *Separation of the proteolytic activity from the phosphofructokinase protein by SDS electrophoresis*

Under denaturing and dissociating conditions as they are provided by SDS electrophoresis a separation of the protease from the subunits of phosphofructokinase may be attained. The protease itself is not inactivated by SDS. Fig. 1 demonstrates the distribution

Table 2
Rechromatography of yeast phosphofructokinase with Sepharose 6 B.

	Specific proteolytic activity (μU/mg protein)
Before chromatography	70
After two times chromatography	61

of protease and total protein after preparative PAA gel electrophoresis in presence of SDS. As revealed by analytical electrophoresis these fractions having the highest proteolytic activity contain only small amounts of phosphofructokinase subunits, whereas

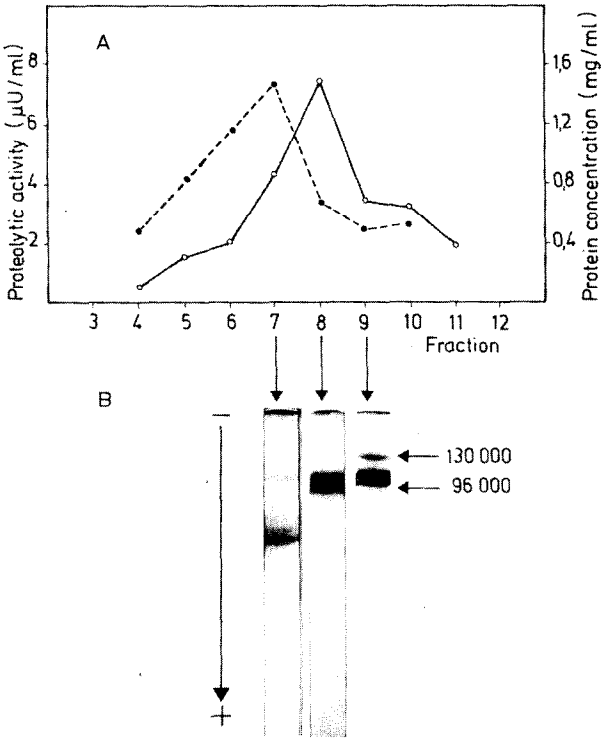

Fig. 1. Preparative and analytical SDS-polyacrylamide gel electrophoresis of yeast phosphofructokinase.
(A) Distribution of proteolytic activity (•--•--•) and phosphofructokinase protein (o—o—o) after electrophoresis in a preparative scale. Starting protein 4 mg. Electrophoresis at 80 mA, 18°, 5 hr.
(B) Fractions number 7, 8 and 9 from the preparative electrophoresis examined by analytical SDS-electrophoresis, 8 mA/tube, 6 hr at 25°.

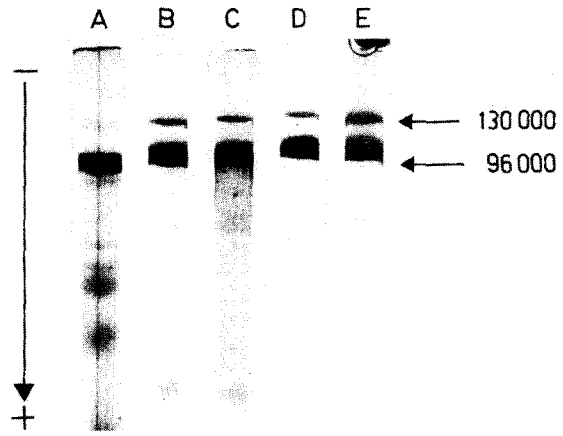

Fig. 2. SDS-polyacrylamide gel electrophoresis of yeast phosphofructokinase after dialysis against F-6-P, ATP and ammonium sulphate. 50 μg yeast phosphofructokinase were dialyzed 24 hr at 3° against 10 mM sodium phosphate buffer pH 7.0.
(A) Buffer only, (B) buffer with 2 mM F-6-P, (C) buffer with 2 mM ATP, (D) buffer with 1.5 M ammonium sulphate. Following redialysis 12 hr against the same buffer containing 1% SDS and 1% 2-mercaptoethanol, (E) SDS-electrophoresis of 50 μg phosphofructokinase before dialysis. Electrophoretic conditions see fig. 1 B.

those fractions containing most of the phosphofructokinase subunits possess very low proteolytic activity. Apparently under these conditions the intermolecular forces between both enzyme proteins are weakened to such an extent that the protease is detached from the phosphofructokinase moiety.

Similar results have been published by Rustum et al. [10] with yeast hexokinase.

3.3. *Effects of substrates and ammonium sulphate on degradation of phosphofructokinase*

F-6-P and ATP as well as ammonium sulphate are able to suppress the attack of phosphofructokinase by the adsorbed protease. This may be shown by dialysis of the enzyme in presence and absence of these compounds and pursuing their effects on subunit composition of phosphofructokinase by SDS electrophoresis (fig. 2). Without these substances the larger subunit of the enzyme disappears leaving only the 96 000 subunit. Simultaneously, smaller peptidic compounds appear which represent evidently degradation products of the larger subunit. In presence of either F-6-P or ATP or ammonium sulphate, respectively, however, the 130 000 subunit is much more stable. The respective electropherograms show only slight variations in the subunit distribution before and after dialysis. Evidently these compounds cause a peculiar conformational state of phosphofructokinase resulting in its greater stability against proteolytic attack. These effects may possibly explain the long known stabilizing effects of substrates as well as of ammonium sulphate (already found by E. Negelein [11] in 1936), on this enzyme.

It should be stressed that these substances are also able to influence the tryptic attack of yeast phosphofructokinase [12, 13].

Thus it seems that the large subunit of phosphofructokinase is more sensitive against proteolytic attack than the small one. This may be the reason why commercial preparations of yeast phosphofructokinase, as well as those which have been prepared by more time consuming methods [1, 3] are composed only by subunits of the 96 000 kind. The 130 000 subunits are evidently proteolytically degraded to the 96 000 ones.

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