# ISOLATION, PURIFICATION AND CHARACTERIZATION OF WHEAT GERM HEXOKINASES

J.C. MEUNIER, J. BUC and J. RICARD

*Laboratoire de Physiologie Cellulaire Végétale associé au C.N.R.S., Centre Universitaire de Marseille-Luminy, Marseille, France*

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## 1. Introduction

During the last few years much work has been performed on the structure of yeast hexokinases [1–8]. Evidence obtained for a subunit structure of these enzymes shows that hexokinases exist as a tetramer [1, 2, 4, 5, 7], or as a dimer [8], of molecular weight approximately 98,000, which can dissociate into two subunits (molecular weight about 49,000). Equilibrium dialysis measurements [9] have shown that each subunit is able to bind one molecule of glucose. However, our knowledge concerning the hexokinases of higher plants is still in its infancy. Until now, only crude preparations have been obtained [10]

The aim of the present work was to purify, for the first time, wheat germ hexokinases and to determine some of their physico-chemical characteristics.

## 2. Methods

Hexokinase activity is determined following the method of Slein et al. [11]. Protein concentration is estimated by the technique of Lowry et al. [12]. Contaminating proteases present at various steps of the purification procedure are determined by the Kunitz method [13] as modified by Laskowsky [14]. Polyacrylamide gel electrophoresis (polyacrylamide gel concentration 7%) is carried out by the method of Davis [15]. Hexokinase activity is detected by the coupled assay of Katzen and Schimke [16]. Protein bands are stained with Amido black. Representative Schlieren patterns of the wheat germ isohexokinases have been obtained with a Spinco model E analytical centrifuge. Centrifugations were performed at 59,780 rpm (bar angle 60°) in $5 \times 10^{-2}$ M phosphate buffer, pH 7. Molecular weights were determined by sieving through either a Sephadex G-100 column (1.6 cm × 110 cm) or a Sephadex G-200 column (1.6 cm × 110 cm). Amino acid analyses were performed with a Beckman Unichrom analyzer.

## 3. Experimental results

Extraction of the hexokinase is effected at 2° by shaking 200 g of wheat germ, for 30 min in 900 ml of phosphate buffer $5 \times 10^{-2}$ M, pH 7.2, containing 0.06% cysteine. Ammonium sulfate precipitation is effected between 50 and 70% saturation. The precipitate containing hexokinase activity is centrifuged then dialyzed. The enzyme preparation thus obtained is applied under gravity to a DEAE-cellulose column equilibrated with a $5 \times 10^{-3}$ M phosphate buffer, pH 7 containing $4 \times 10^{-2}$ M glucose and proteins eluted from the column with a KCl gradient. Fractions containing hexokinase activity are submitted to molecular sieving on Sephadex G-100. Proteins are washed with $5 \times 10^{-2}$ M phosphate buffer, pH 7 containing $10^{-1}$ M KCl and $10^{-1}$ glucose (fig. 1). Two elution peaks (A and B) of hexokinase activity are observed (fig. 1). If the fractions containing these peaks are submitted to a new molecular sieving on Sephadex G-100, almost symmetrical profiles superimposable upon the elution peaks of fig. 1 are obtained.

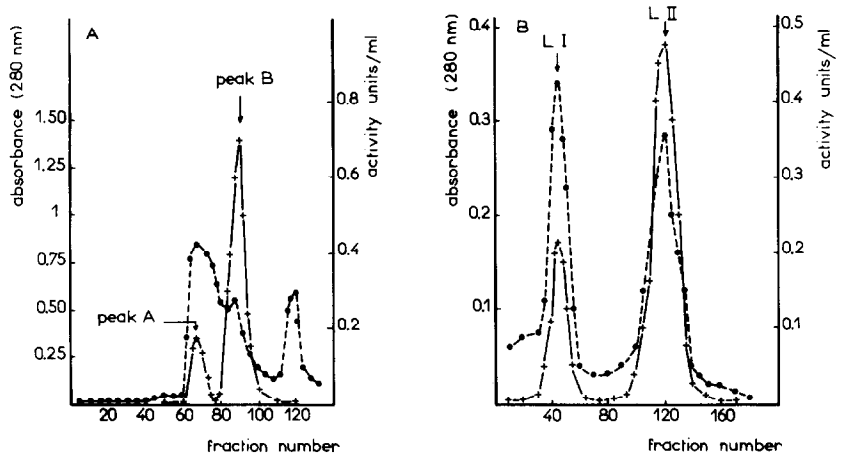


Fig. 1. (A) Molecular sieving on Sephadex G-100 "superfine". The enzyme preparation is applied after concentration, on a Sephadex G-100 column equilibrated in $5 \times 10^{-2}$ M phosphate buffer pH 7, containing 0.1 M potassium chloride and 0.1 M glucose. The flow rate is 5 ml/hr and the fraction volume is 3 ml per tube. (B) DEAE-cellulose chromatography of the fractions contained in the B peak. After washing the column with a succinate buffer ($5 \times 10^{-3}$ M, pH 5.8) containing glucose ($4 \times 10^{-2}$ M), elution is effected with a pH gradient from 5.8 to 4.5 in a succinate buffer containing $4 \times 10^{-2}$ M glucose. The flow rate is 30 ml/hr and the fraction volume is 3 ml per tube. + —— +, hexokinase activity (U/ml) at 30°; ● - - - - ●, absorbance at 280 nm.

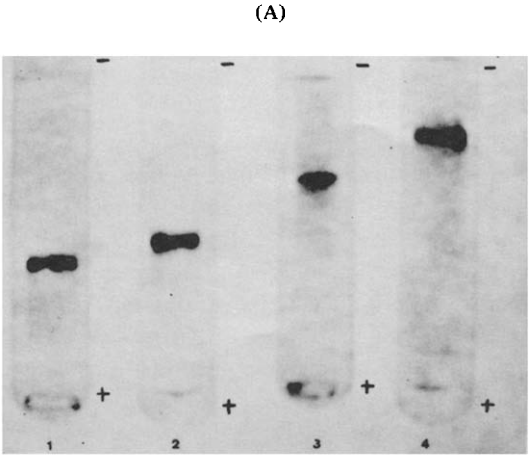


Fig. 2 A. Polyacrylamide gel electrophoresis of wheat germ hexokinases. Enzymes (75 μg) after dialysis against a $3.75 \times 10^{-3}$ M, tris-HCl buffer, pH 7, are subjected to electrophoresis, and stained with Amido-black. (1) "light" I, (2) "light" II, (3) "heavy" I, (4) "heavy" II.

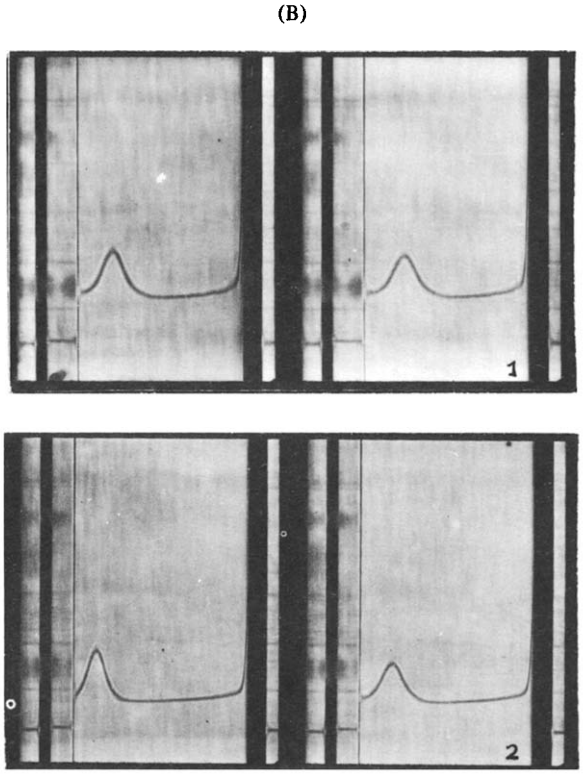


Fig. 2 B. Sedimentation velocity patterns of "light" isohexokinases. (1) "light" I, (2) "light" II. Protein concentration is either 6 mg/ml "light" I) or 6.8 mg/ml ("light" II). Hexokinases are dissolved in a $5 \times 10^{-2}$ M, pH 7 phosphate buffer. Temperature 20°, bar angle 60°.

Table 1
Comparative amino acid composition (moles per mole protein, MW 48,000) of wheat germ isohexokinases $L_I$ and $L_{II}$ and yeast hexokinases $P_I$ and $P_{II}$ [4].

| | Wheat germ hexokinases | | Yeast hexokinases | |
|---|---|---|---|---|
| | $L_I$ | $L_{II}$ | $P_I$ | $P_{II}$ |
| Lysine | 31 | 32 | 33 | 32 |
| Histidine | 8 | 7 | 8 | 5 |
| Arginine | 17 | 17 | 15 | 17 |
| Aspartic acid | 48 | 49 | 49 | 49 |
| Threonine | 22 | 20 | 26 | 26 |
| Serine | 28 | 26 | 22 | 21 |
| Glutamic acid | 55 | 55 | 45 | 48 |
| Proline | 19 | 26 | 21 | 24 |
| Glycine | 46 | 45 | 37 | 36 |
| Alanine | 43 | 34 | 30 | 30 |
| ½ Cystine | 4 | 3 | 4 | 3 |
| Valine | 36 | 36 | 26 | 21 |
| Methionine | 7 | 6 | 10 | 10 |
| Isoleucine | 22 | 21 | 28 | 32 |
| Leucine | 39 | 40 | 39 | 32 |
| Tyrosine | 13 | 11 | 15 | 14 |
| Phenylalanine | 16 | 16 | 16 | 21 |

The enzyme preparation contained in the B peak is dialyzed two hr against a $5 \times 10^{-3}$ M succinate buffer, pH 6, containing $4 \times 10^{-2}$ M glucose. The dialyzate is applied to a DEAE-cellulose column equilibrated with $5 \times 10^{-3}$ M succinate buffer, pH 5.8, containing $4 \times 10^{-2}$ M glucose. Hexokinases are eluted from the column with a pH gradient, from 5.8 to 4.5, generated by a constant volume method (fig. 1). In this way, two elution peaks, I and II, containing isohexokinases $L_I$ ("light") and $L_{II}$ ("light" II), respectively, are obtained. In the same way (DEAE-cellulose chromatography, elution with a pH gradient) it can be shown that fractions of elution peak A also contain two isohexokinases: $H_I$ ("heavy" I) and $H_{II}$ ("heavy" II).

The four isohexokinases $H_I$, $H_{II}$, $L_I$ and $L_{II}$, obtained in this way, are highly purified. They are homogeneous on polyacrylamide gel electrophoresis (fig. 2). Sedimentation velocity patterns of the two forms $L_I$ and $L_{II}$ are symmetrical (fig. 2). We have not been able to obtain sufficient amounts of $H_I$ and $H_{II}$ to test their homogeneity on analytical centrifugation.

Molecular weights of isohexokinases $L_I$, $L_{II}$, $H_I$, $H_{II}$ have been determined either by molecular sieving on Sephadex G-100 ($L_I$, $L_{II}$) or on Sephadex G-200 ($H_I$, $H_{II}$). They are very close to 50,000 and 100,000, respectively. Variation of ionic strength, pH, enzyme or glucose concentration does not seem to produce aggregation of "light" forms, or dissociation of "heavy" forms.

Amino acid composition of the two isoenzymes $L_I$ $L_{II}$ is given in table 1. A close similarity exists between their composition and that found by Gazith et al. [4] for yeast hexokinases $P_I$ and $P_{II}$ (table 1).

In addition to these native isoenzymes, other hexokinase forms appear in various experimental conditions, during the purification procedure. If for instance the extract after ammonium sulfate fractionation and dialysis is kept for several hours at 35° before DEAE-cellulose chromatography, two other acidic hexokinase forms are present in addition to "L" and "H" forms. If, however, the extract is immediately chromatographed, these acidic hexokinases do not appear. These results are clearly in agreement with the idea that these forms are not native enzymes, but artifacts due to a partial proteolysis. Similar results with yeast hexokinases have been obtained by various investigators [6, 17].

## 4. Discussion

From the results of table 1, it appears that the amino acid compositions of the two forms $L_I$ and $L_{II}$ are very similar. However, enzyme $L_I$ contains 19 proline residues and 43 alanine residues as compared with 26 proline residues and 34 alanine residues in $L_{II}$. Also, there is a marked similarity between the amino acid compositions of wheat germ enzymes and yeast enzymes (table 1). Some differences are observed in the number of residues of glutamic acid, glycine, and valine.

In the case of yeast enzyme, it has been observed [4, 6, 17] by DEAE-cellulose chromatography that form $P_I$ is more basic than $P_{II}$. However, $P_I$ behaves as the more acidic form by polyacrylamide gel electrophoresis [4, 6, 17]. This reversed mobility has not been explained [4, 17]. Similar results are obtained in the case of wheat germ hexokinases $L_I$ and $L_{II}$. By chromatography, $L_I$ is more "basic" than $L_{II}$, but more "acidic" by gel electrophoresis (figs. 1 and 2).

The chromatographic behavior of wheat germ enzyme

$H_I$, $H_{II}$, $L_I$ and $L_{II}$ is similar to that of yeast enzymes of the "P" type [4, 6, 17]. In both cases these hexokinases are probably native enzymes.

It has already been shown by various workers [2, 3, 6] that glucose, high ionic strength, and neutral or basic pH, cause dissociation of yeast hexokinases (MW 96,000) into two subunits (MW 48,000). In the absence of glucose, at low ionic strength and low pH, one can observe the association of the two subunits. Phosphate and low concentrations of enzyme also favor the dissociation process [3, 6].

In the case of wheat germ hexokinases we have never been able to observe the reversible interconversion:

$$\text{L ("light" form)} \rightleftharpoons \text{H ("heavy" form)}$$

in experimental conditions which permit interconversion in yeast hexokinases.

Moreover, it is impossible to separate $L_I$ from $H_I$, and $L_{II}$ from $H_{II}$ on DEAE-cellulose chromatography. Therefore, one must conclude that the charges of $L_I$ and $H_I$ on one hand, $L_{II}$ and $H_{II}$ on the other hand, are nearly identical.

The above results clearly show that substantial differences occur between yeast and wheat germ hexokinases. It is possible that differences also occur in the mechanisms of transphosphorylation reactions catalyzed by these enzymes.

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