THREE FORMS OF ENOLASE SEPARATED BY COUNTER-CURRENT DISTRIBUTION

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1. Introduction

The existence of enzymes in multiple forms (isoenzymes) is well documented [1]. The enzyme enolase (EC 4.2.1.11) has been shown to be electrophoretically heterogeneous [2]. At least 3 forms exist in yeast [3, 4] and in muscle of several species of trout [5]. Some have proposed that this may be an artifact resulting from the preparation of the enzyme [2, 6].

The separation of the enzymes of the glycolytic system in bakers yeast has been studied by extraction as well as counter-current distribution using aqueous two-phase systems containing polyethylene glycol carrying ionisable groups and dextran [7]. In this communication we present results that demonstrate the presence of 3 forms of enolase in bakers yeast.

2. Materials and methods

Dextran used was "Dextran 500" (M.W. = 5×10^5) obtained from Pharmacia, Uppsala, Sweden. Trimethylamino-polyethylene glycol, TMA-PEG, was prepared from polyethylene glycol "Carbowax 6000" ($M_n = 6 \times 10^3$) obtained from Union Carbide, USA [7]. This contains 0.10 meq TMA groups per g. Enolase, type III, from yeast, was obtained from Sigma Chemical Co., St. Louis, USA. The salts and other chemicals used were of analytical reagent grade.

2.1. *Assay for enolase*

The enolase activity was measured according to the method of Warburg and Christian [8]. 100 µl of the solution to be assay was mixed with 2 ml of the substrate solution having the following composition: 2.5×10^{-3} M sodium DL-2-phosphoglycerate prepared according to Kiessling [9], 5×10^{-5} M EDTA 4×10^{-3} M $MgSO_4$, 0.02 M potassium phosphate buffer, pH 6.8. The change in absorbance at 240 nm and 25° was measured using 1 cm cells in a Unicam SP 800 spectrophotometer and recorded on a Servogor recorder. Activity is expressed as the initial change in $A_{240}^{1\,cm}$ per min.

2.2. *Preparation of yeast extract*

Fresh bakers yeast obtained from Jästbolaget (Sollentuna, Sweden) was mixed with crushed dry ice in 1:1 ratio and the mixture was homogenized in a Turmix mixer. The homogenate was spread on a plastic plate to allow the evaporation of dry ice. The yeast homogenate was then centrifuged for 20 min at 15,000 *g*. The turbid supernatant was collected and recentrifuged for 60 min at 160,000 *g*. The clear supernatant so obtained was dialysed for 5 hr at 10° against 14 or 20 mM K_2HPO_4–KH_2PO_4 buffer solution of the required pH, which was 5.5 in the case of extraction experiments and 6.5 in the case of counter-current distribution. All the other steps were carried out at 3°.

2.3. *Preparation of enolase from bakers yeast*

The yeast extract obtained as under sect. 2.2 was subjected to fractionation steps according to Malmström [2]. The precipitate obtained in the last ethanol precipitation step was dialysed against 14 mM phosphate buffer at 3°. The dialysate after removal of precipitated material was used in counter-current distribution experiments.

2.4. *Preparation of two-phase system*

The two-phase systems used in these experiments contained 6.6% (wt/wt) dextran 500, 6.4% (wt/wt) TMA–PEG and 5 mM potassium phosphate buffer at the appropriate pH. Phase systems were prepared by mixing required quantities of stock solutions of the polymers and the dialysed yeast extract or enolase solution. Blank systems were prepared by adding an equivalent quantity of buffer solution in place of the yeast extract.

2.5. *Extraction with two-phase system*

The extraction of enolase as a function of pH was studied as follows: A phase system containing 25% (wt/wt) of dialysed yeast extract was prepared at pH = 5.5 and the pH of the system was raised in discrete steps by careful addition of 2 M NaOH under stirring. At each step the system was centrifuged to speed up the phase separation. Samples were withdrawn from the upper phase and the concentration of enolase measured in terms of its enzyme activity. The pH in each step was measured with aid of a glass electrode that was directly immersed in the upper phase. In calculating the per cent of enolase activity in the upper phase at each pH, corrections were made for the loss in enzyme activity due to withdrawal of samples from the upper phase for analysis. The total amount of enolase in the system was determined by taking an assay of the mixed system at the starting pH. The entire procedure required not more than 2 hr.

2.6. *Counter-current distribution*

The counter-current distribution was carried out at pH 6.5. The apparatus used was an automatic thin-layer counter-current distribution apparatus with 60 cavities [10]. The first cavity was filled with 1.4 ml of the phase system containing yeast extract or solution of enolase and the rest of the cavities were filled with 0.7 ml of each of the 2 phases obtained from the blank system. The settling time was 5 min, and the shaking time was 30 sec. The temperature was 20°. After 60 transfers the content of each cavity was diluted with 1.4 ml water, collected and then assayed for enolase activity. The total time required for the counter-current distribution experiment and activity measurements was 10–11 hr.

3. Results and discussion

The extraction profile for enolase as a function of pH is shown in fig. 1, along with results obtained with similar experiments on CO–hemoglobin. It is seen that the curve for enolase consists of 3 distinct steps, which are absent in the case of hemoglobin. In systems containing PEG carrying ionisable groups, the partition of a protein is essentially dependent on charge. There will be an extreme change in partition near the isoelectric point of the protein. Thus, the concentration of hemoglobin in the upper phase changes from 10% to 80% between pH 7–9. By analogy, for a protein which consists of multiple forms which differ in their isoelectric points, the extraction curve would consist of a number of steps each corresponding to one form. By this criteria one can suspect from the extraction profile for enolase that the enzyme consists of at least 3 different forms. The amount of enzyme extracted in the 3 steps are in the proportion 3:3:4.

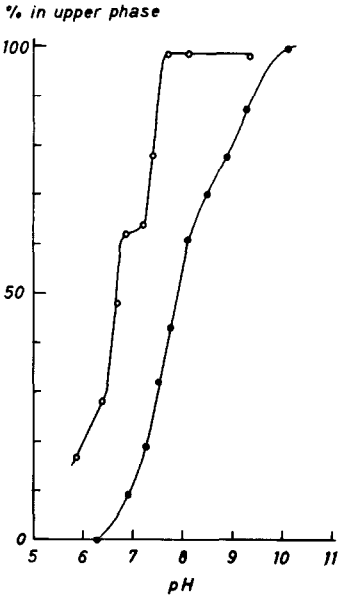

Fig. 1. Extraction profiles as a function of pH for enolase in yeast extract (○—○—○) and CO–hemoglobin prepared from human blood (●—●—●). The ordinate records the amount of protein in the upper phase as per cent of the total amount of that protein in the system. Enolase concentration is measured in terms of activity (see sect. 2.5), and the concentration of CO–hemoglobin in terms of absorbance at 500 nm.

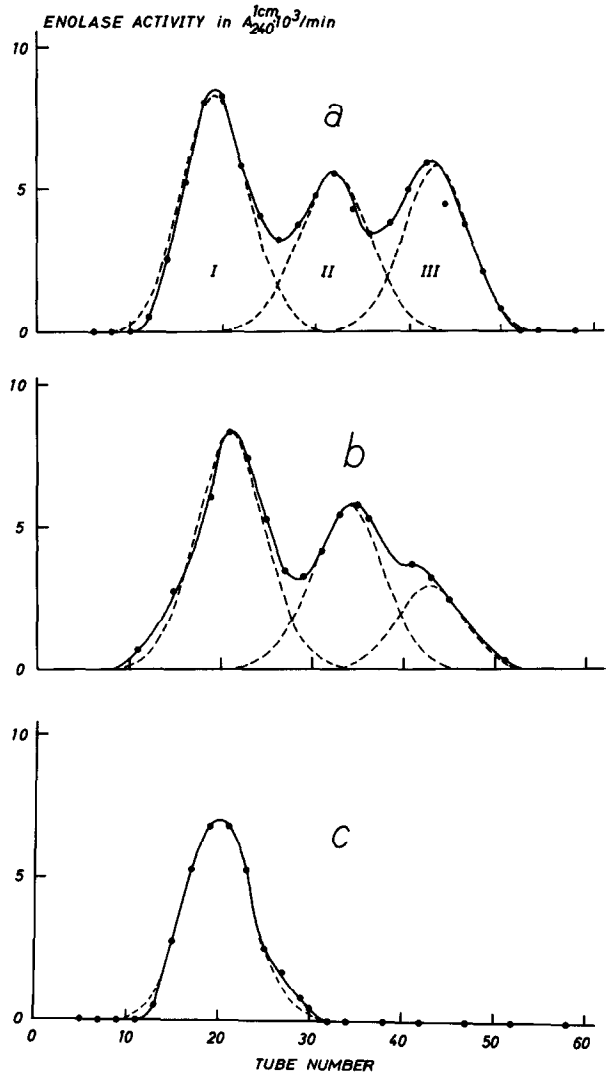

Fig. 2. Counter-current distribution of enolase in aqueous phase systems containing 6.6% (wt/wt) dextran "500", 6.4% (wt/wt) TMA–PEG and 5 mM potassium phosphate buffer. a) Enolase in yeast extract; b) enolase prepared from yeast; c) enolase obtained from Sigma Chemicals Co., USA. See sect. 2.6 for details. (——) Experimental curves; (- - - -) theoretical curves corresponding to each peak.

Table 1
Relative proportions of three forms of yeast enolase obtained in different preparations.

Method	Per cent of component		
	I	II	III
Counter-current distribution of yeast extract.	41	29	30
Counter-current distribution of enolase prepared by the method of Malmström [2].	50	33	17
Electrophoretic separation of yeast enolase. Data from [4].	41	29	20
Electrophoretic separation of yeast enolase. Data from [11].	56	20	24

The results of the counter-current distribution of yeast extract at pH 6.5 are shown in fig. 2a. The enolase activity is distributed over 3 distinct peaks. This again indicates that enolase is not homogeneous, but consists of at least 3 forms which differ in their partition characteristics at the pH used in the experiment. In this case the lower the isoelectric point the higher will be the partition coefficient. Analysis of the 3 experimental peaks has been carried out and the best fitting theoretical curves corresponding to each of the peaks are also shown in fig. 2a. The counter-current distribution of enolase prepared in this laboratory from yeast, shown in fig. 2b, also yields 3 peaks, while only a single peak is obtained with the commercial preparation, fig. 2c. This peak corresponds to peak I in fig. 2a and 2b. According to manufacturers this product is prepared essentially per Westhead and McLain [6], whose method is designed to yield only a single fraction.

The relative proportions of the 3 forms have been calculated from the area under the peaks. This is with the assumption that the concentration of enolase is proportional to its activity at each point of the counter-

current distribution curve. The results are given in table 1. These agree with the results obtained in the extraction experiments, sect. 2.5. The comparatively longer time required for a counter-current distribution experiment does not therefore seem to influence the relative proportions of the 3 forms. The relative proportions of 3 forms of enolase obtained by gel electrophoresis reported by Pfleiderer et al. [4] and Geidan and Zhagat [11] are included for comparison. Since the molecular weight of the 3 forms of enolase is the same [2, 4], their separation by counter-current distribution or by gel electrophoresis will depend on the charge carried out by each form under the conditions of the experiment. Thus, the 3 forms corresponding to the peaks I, II and III in the counter-current distribution curves, fig. 2, will be in order of negative charge or decreasing isoelectric points. When separated by gel electrophoresis, the form with lowest isoelectric point is most likely to be the one nearest the anode. On this basis we assume that the 3 enolase forms referred to as I, II and III in this paper correspond, respectively, to the forms III, II and I of [4], and 3, 2 and 1 of [11]. The fractionation of biological substances by multistage distribution between the phases of aqueous polymer systems is a very mild method [10]. It involves neither precipitation nor adsorption at active surfaces, e.g. ion exchangers. It is most likely that the 3 forms detected with this method are orginally present in the cell. The 3 forms of enolase may be more or less sensitive towards treatment such as precipitating agents, pH or adsorbents. Further, their solubility under a given condition may not be the same, since the 3 forms differ in their isoelectric points [3]. These 2 factors may cause a change in the relative proportions of the forms in purified enolase as compared to the state in the cell. This may be avoided if the 3 forms of enolase are first isolated by counter-current distribution method used in this work. Each form can then be further purified by conventional methods and characterized. Such work is in progress.

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