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ISOLATION AND PURIFICATION OF A REGULATING COFACTOR OF THE PENTOSE-PHOSPHATE PATHWAY

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Summary.

Cofactors that counteract the inhibition of glucose 6-phosphate dehydrogenase by NADPH, in the presence of GSSG, have been isolated and purified from mussel hepatopancreas and rat liver by gel filtration on Sephadex G-50 and polyacrylamide gel electrophoresis. The cofactors from mussel hepatopancreas and rat liver differ in apparent molecular wight, having values of 15,000 and 10,000 daltons, respectively. Both cofactors undergo inactivation by trypsin, while RNase and DNase have no effect on their activity.

It has been established by various authors (Cahil et al., 1958; Williamson et al., 1971) that the velocity of the pentose-phosphate cycle is controlled at the glucose 6-phosphate dehyddrogenase step. There is total inhibition of glucose 6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase when the NADPH/$NADP^+$ ratio approaches 9 (Eggleston & Krebs, 1974; Silva Pando, 1976). The ratio between these nucleotides calculated for the cytoplasm of rat liver is of the order of 100 (Veech, 1968; Veech et al., 1969). Thus one might conclude that it is impossible for the pentose-phosphate pathway to operate in vivo.

Eggleston & Krebs (1974) found that GSSG* at physiological concentrations is able to counteract the inhibition of glucose 6-phosphate dehydrogenase by NADPH. They likewise showed that for GSSG to be effective required the presence of a highly unstable unidentified cofactor. Rodríguez-Segade et al. (1978) have found that in the hepatopancreas of mussel (Mytilus edulis L.) GSSG has a similar effect on glucose 6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase, which is more pronounced on the latter particulary at a high ($NADPH/NADP^+$) ratio. The present paper

* GSSG: Oxidized glutathione.

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describes the isolation and purification by means of gel filtration on Sephadex G-50 and preparative electrophoresis on polyacrylamide gels, of the cofactor that, with GSSG, contributes to the regulation of the oxidative enzymes of the pentose phosphate pathway in mussel hepatopancreas and rat liver.

Materials and Methods.

To obtain crude enzyme preparationsof glucose 6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase, weighed samples of hepatopancreas from Mytilus edulis L. (N.W. Spain) or rat liver were homogenized with 3 vol of ice-cold 0.1 M potassium phosphate buffer, pH 7.4 containing 1 mM EDTA. After centrifugation at 27,000 x g for 30 min at 2°C the supernatant solutions were used for isolation of cofactor.

Gel filtration on Sephadex G-50 was used to isolate the cofactor from the tissue homogenates and to determine its molecular weight (Andrews, 1964). The cofactor was purified to homogeneity by preparative electrophoresis on polyacrylamide gels (Davis, 1964).

Amicon Centriflo membrane cones 224-CF-50A (supplied by Amicon N.W., Oosterhout (N.B.), Holland) were soaked for 2 h in water before use. Tissue homogenates of various concentration (5 ml) were centrifuged in the cones for 0.5-1 h at 1,000 g and 4°C. Some tissue preparations were treated in a Minicon B-15 macro-solute concentrator (Amicon N.V.). Protein concentration was determined according to Lowry et al. (1951).

The following reaction mixture was used to determine the effect of the cofactor upon glucose 6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase: 60 mM Tris HCl buffer, pH 7.4; 5 mM $MgCl_2$, 0,33 mM $ZnSO_4$, 0,08 mM NADPH, 0,02 mM $NADP^+$, 1 mM GSSG (this latter added to only one pair of cuvettes). Control blanks without 6-phosphogluconate or glucose 6-phosphate were run simultaneously. The effect of the cofactor was determined as the percentage activation observed when the cofactor was added in the presence of GSSG.

The effect of the trypsin, RNase and DNase upon the activity of the cofactor was carried out by incubation with these enzymes for 40 minutes at room temperature. Then the cofactor effect was determined as indicated above.

When trypsin was used, trypsin inhibitor (Tipe 1-S from Soybean; Sigma) was added before the cofactor activity assay.

Results and Discussion.

As observed with rat liver (Eggleston & Krebs, 1974) dialysis of mussel hepatopancreas homogenates eliminates the reversal by GSSG of the inhibition of glucose 6-phosphate dehydrogenase and also of 6-phosphogluconate dehydrogenase by NADPH. Complete loss of the GSSG effect resulted after dialysis for 24 h.

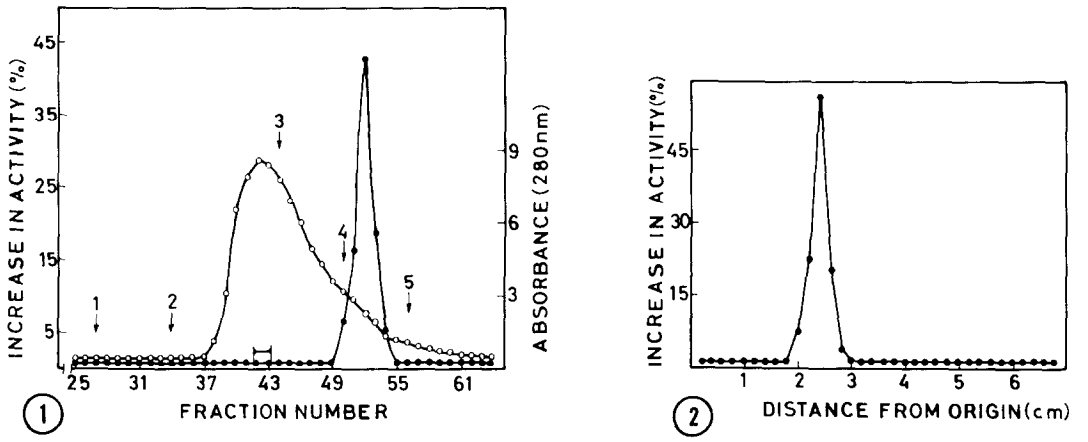

Figure 1. Sephadex G-50 chromatography of mussel hepatopancreas homogenate. 25 ml of homogenate (about 300 mg) were chromatographed on a 2.5 x 65 cm Sephadex G-50 column equilibrated and eluted with 50 mM Tris. HCl buffer, pH 7.0, and fractions of 3 ml collected. Cofactor activity (•), expressed as the percentage of the increase in the activity of glucose 6-phosphate dehydrogenase from mussel hepatopancreas (see Materials and Methods), and absorbance at 280 nm (o) were determined in each fraction. Arrows and numbers indicate the elution positions for markers used in the calibration of the column: (1) bovine serum albumin (M.W. 67,000); (2) ovalbumin (M.W. 45,000); (3) chymotrypsinogen (M.W. 25,000); (4) myoglobin (M.W. 17,800); (5) cytochrome c (M.W. 12,400).

Figure 2. Preparative polyacrylamide gel electroforesis of cofactor from mussel hepatopancreas. Aliquots of about 0.8 mg of protein, from the fractions with maximum cofactor activity in the Sephadex G-50 column, were loaded on 1 x 7 cm gels. The gels were prepared and the electrophoresis carried out according to the procedure of Davis (1964) at 4°C. The gels were cut into 2 mm slices and extracted with 0.1 M phosphate buffer for 1 h at 4°C. The cofactor activity of each slice was determined as indicate in Figure 1.

Treatment of the mussel hepatopancreas homogenates using Amicon membranes, which separate components of molecular weight

below 50,000, yield active solutions of cofactor. The enzymatic activity of both dehydrogenases is retained within the Amicon membranes, and the percentage activation of GSSG on this fraction is approximately 11%. The addition of 0.1 ml of the Amicon ultrafiltrate to the enzyme solution increased the percentage activation by GSSG to 45% in the case of glucose 6-phosphate dehydrogenase. Treatment using Minicon cells, which eliminate components of molecular weight below 15,000 daltons, results in a substantial decrease (nearly 20%) in the effectiveness of the cofactor present in the Amicon ultrafiltrates. These results suggest that the cofactor has a molecular weight close to the limit of molecular exclusion of Minicon B-15 cells.

For the isolation and purification of the cofactor, the homogenate was subjected to gel filtration on Sephadex G-50 and preparative electrophoresis on polyacrylamide gels. Approximately 25-30 mol of hepatopancreas homogenate, representing about 300 mg of protein, were loaded on Sephadex G-50 colum (Fig. 1). The enzymatic activity of glucose 6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase was determined in each fraction collected. The maximum activity of these enzymes is corresponding with the maximum of protein and fractions designated by the bar, shown of Fig. 1, were used as source of both enzymes. These fractions contained no cofactor activity. A sharp peak of cofactor activity emerged in the fractions corresponding to a molecular weight of 15,000. The fractions with greatest cofactor concentration did not exhibit gluthathione reductase activity.

The effect of cofactor was also tested with 6-phosphogluconate dehydrogenase. It was found that only those fractions affecting glucose 6-phosphate dehydrogenase activity enhance 6-phosphogluconate dehydrogenase.

From the Sephadex G-50 fraction presenting greatest cofactor effect (fraction 52 in Fig. 1), the cofactor was isolated by preparative electrophoresis on polyacrylamide gels. The gels were cut into 2 mm slices and each slice extracted with 0.1 M potassium phosphate buffer, pH 7.0 and tested for cofactor activity (Fig. 2). An aliquot of the peak with cofactor activity was submitted to analytical electrophoresis and after staining with coomasie blue, the cofactor could be observed as a homogeneous band (Fig. 3).

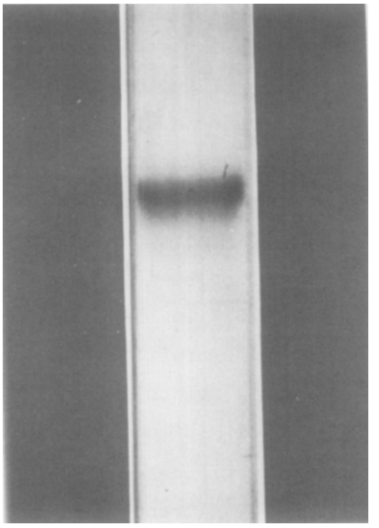

Figure 3. Electrophoresis on polyacrylamide gels of cofactor from mussel hepatopancreas. An aliquot of cofactor (50 µg), isolated by preparative polyacrylamide electrophoresis, was loaded on a 0.5 x 7 cm gel. The electrophoresis was carried out according to the procedure of Davis (1964). The gels were stained with 0,25% Coomasie blue in 12.5% trichloroacetic acid for 2 h.

The same procedure was employed to isolate the cofactor from rat liver, using a homogenate prepared as indicated for mussel hepatopancreas. Gel filtration on Sephadex G-50 yield a sharp peak of cofactor activity emerging in fractions corresponding to a molecular weight of 10,000 daltons (data not shown). Again the cofactor was active with both glucose 6-phosphate and 6-phosphogluconate dehydrogenase fron rat liver. As in the hepatopancreas procedure, the cofactor was purified by preparative polyacrylamide gel electrophoresis (data not shown). Analytical electrophoresis shows the rat liver cofactor as a homogeneous band (Fig. 4).

In order to characterize the cofactors, incubations of both, obtained by preparative electrophoresis, were carried out with trypsin, RNase and DNase. The results are presented in Table I.

On the basis of these results the cofactors from mussel hepatopnacreas and rat liver resemble to be polypeptides with

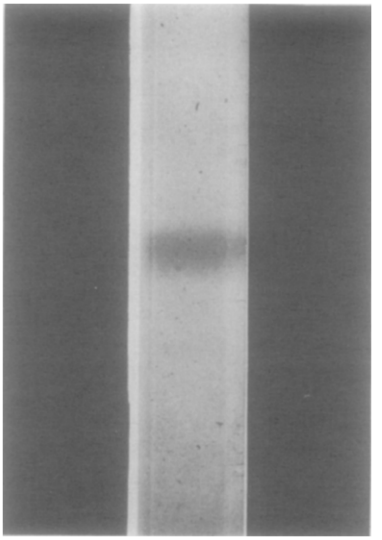

Figure 4. Electrophoresis on polyacrylamide gels of cofactor from rat liver. An aliquot of cofactor (50 µg), isolated by preparative electrophoresis, was loaded on a 0.5 x 7 cm gel. The electrophoresis was carried out in the same conditions as indicated in Fig. 3.

TABLE I. Effect of trypsin, RNase and DNase upon the activity of cofactor isolated from mussel hepatopancreas and rat liver.

	Increase in activity (%)	
	Hepatopáncreas	Liver
Cofactor alone	30	27
Cofactor + trypsin	0	0
Cofactor + RNase	30	27
Cofactor + DNase	30	27

About 600 µg of cofactor isolated from mussel hepatopancreas and rat liver by preparative electrophoresis, were incubated with trypsin (12 µg), RNase (10 µg) or DNase (10 µg) for 45 min at room temperature. Then the cofactor activity was determined as described under Materials and Methods, by using glucose 6-phosphate dehydrogenase isolated from the Shepadex G-50 columm from mussel hepatopancreas or rat liver (see text) for the respective cofactor. When trypsin was used, trypsin inhibitor (12 µg) was added after incubation.

apparent molecular weight 15,000 and 10,000 daltons respectively, and we may suppose that regulation of the pentose phosphate pathway is exercised by the system NADPH-GSSG-cofactor, which may be a general phenomenon in animal tissues.

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