Studies on Membranes Isolated from Extracts of *Fusarium culmorum*

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SUMMARY

A method was developed for the fractionation of *Fusarium culmorum* homogenates in order to obtain purified cytoplasmic membranes. The fractions obtained were characterized by studying their ultrastructure and enzymic properties. Since ATPase is an enzyme present in the cytoplasmic membrane, we studied the levels of this activity in our fractions as an indication of the presence of cytoplasmic membrane fragments. Since this activity is also present in mitochondria, we determined the levels of fumarase, a mitochondrial enzyme, and took the ratio of the two activities as an index of the purity of the membrane preparations.

The sediment obtained by centrifuging the cell-free extract at 40000 *g* was fractionated in a discontinuous sucrose gradient. This led to three types of fractions. The most dense had a high ATPase/fumarase ratio and its ultrastructure showed that it contained membrane fragments having a triple-layered structure. We concluded that this fraction was rich in cytoplasmic membrane fragments; it was clearly distinguishable from the intermediate and less dense fractions. These latter fractions had a much lower ATPase/fumarase ratio and, judging by their ultrastructure, they were respectively a mitochondrial fraction and a fraction consisting of vesicles probably related to the endoplasmic reticulum. Analysis of the cytoplasmic membrane-rich fraction showed that it consisted of protein, lipid and 30 % carbohydrate

INTRODUCTION

The structure and function of cytoplasmic membranes are the subjects of many current investigations in biology which depend on the development of methods for the isolation of pure membrane preparations. In eucaryotes there are intracellular membrane systems, structures similar to the cytoplasmic membrane and possibly related to it. Their presence complicates the isolation of cytoplasmic membranes since the isolation procedure used has to be selective enough to isolate cytoplasmic membranes uncontaminated by other membranous materials.

Few studies have been carried out on the plasmalemma of fungi. They were almost exclusively limited to several species of yeast, and made use of two methods for the isolation of plasmalemma. Boulton (1965) and Longley, Rose & Knights (1968), working with *Saccharomyces cerevisiae*, García-Acha *et al.* (1966) with *Fusarium culmorum*, and García Mendoza & Villanueva (1967) and Schwencke, Krsulovic, Rojas & Farias (1972) with *Candida utilis*, made membrane preparations from protoplasts, by producing protoplasts by enzymic digestion of the cell wall, followed by osmotic lysis of the protoplasts and finally the isolation of membranes by differential centrifugation of the lysates. On the other

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hand, Matile, Moor & Mühlethaler (1967) and Matile (1970) reported the isolation of membranes from *S. cerevisiae* by homogenizing the cell by mechanical breaking and subsequently fractionating the homogenates by differential and density-gradient centrifugation.

The chemical compositions of the preparations obtained by these two methods are not the same. A much higher carbohydrate content has been found in membranes isolated by homogenization than in membranes isolated from protoplasts. The discrepancies reflect the complexity of the problem of the isolation of membranes. There is a need for the development of methods for the fractionation of the cell components and for a very careful examination by biochemical and structural methods of the fractions that are obtained, in order to determine the components of each fraction.

We describe the methods and conditions that we used for the fractionation of *Fusarium culmorum* extracts and the analysis that we made in order to achieve the purification of cytoplasmic membrane fractions.

METHODS

Organism and growth conditions. *Fusarium culmorum* 2148 of the Colección Española de Cultivos Tipo (CECT) was used. Stock cultures were maintained on slants of GAE medium (García-Acha & Villanueva, 1963). The fungus was grown with vigorous aeration in 300 ml Erlenmeyer flasks, containing 70 ml of GAE medium previously inoculated with a spore suspension. Cells were harvested in the exponential phase of growth before sporulation had taken place, and then washed with distilled water.

Biochemical determinations. The specific activities of ATPase and fumarase and the protein concentration were determined for different particle suspensions. For the ATPase assay 1 ml of the reaction mixture contained (μmol): tris-HCl (pH 7.2), 100; ATP, 2; KCl, 7.5; NaCl, 7.5; MgCl_2 , 4; and samples of the corresponding particle suspension. The mixture was incubated for 30 min at 30 °C and the reaction stopped by addition of 1 ml of 20 % (w/v) trichloroacetic acid. The precipitated protein was separated by centrifugation and the supernatant assayed for liberated inorganic phosphate by the method of Fiske & Subbarow (1925).

Corresponding controls were run in each assay to determine non-enzymic degradation of ATP and liberation of inorganic phosphate in the absence of ATP. One unit of enzyme will liberate 1 μmol of inorganic phosphate in 60 min under the assay conditions described above.

Fumarase was assayed spectrophotometrically by the method of Hill & Bradshaw (1969). The substrate, L-malate, was dissolved in 0.05 M-phosphate buffer (pH 7.3) to a concentration of 0.05 M. Initial variations in absorption at 250 nm were determined. The number of units of activity for a sample was defined as a thousand times the initial rate of change in extinction/10 s.

The protein concentration of the particulate fractions was determined spectrophotometrically by the method of Layne (1959). Specific activities of both enzymes were expressed as no. of units/mg protein.

When the chemical composition of some lyophilized fractions was examined, the protein content was determined by the method of Lowry, Rosebrough, Farr & Randall (1951), the carbohydrate content by the anthrone method (Trevelyan & Harrison, 1952), and the lipid content gravimetrically in extracts obtained by the method of Folch, Lees & Sloane-Stanley (1957). For the qualitative analysis of carbohydrate, a few milligrams of the sample were hydrolysed by treatment with 25 N- H_2SO_4 at 4 °C for 12 h, followed by 1 N- H_2SO_4 at

105 °C for 8 h. The acid was neutralized with $\text{Ba}(\text{OH})_2$, the BaSO_4 precipitate was removed and samples were chromatographed on Whatman no. 1 paper. The solvent mixture was ethyl acetate:pyridine:water, 8:2:1 (v/v) and the sugar spots were detected by subsequent treatments with AgNO_3 diluted in aqueous acetone, NaOH in aqueous ethanol, and finally with 5 % (w/v) $\text{Na}_2\text{S}_2\text{O}_3$. As a specific stain for fructose, a 1 % solution of α -naphthol in ethanol:phosphoric acid 10:1 (v/v) was used.

Electron microscopy. For the ultrastructural study of the particle suspensions of fractions from the sucrose gradients, enough 50 % glutaraldehyde was added to each fraction to give a final concentration of 4 %. After 2 h the particles were sedimented by centrifugation at 150000 g for 15 min, washed twice with 0.4 M-sucrose in 0.1 M-phosphate buffer (pH 7.0) and postfixed with 2 % (w/v) OsO_4 in the same buffer. Best results were obtained when this fixation was prolonged for 12 h. Fixed samples were dehydrated through 25, 50, 75 and 100 % (v/v) acetone. During dehydration the material was stained overnight with 2 % (w/v) uranyl acetate dissolved in the 75 % acetone solution. The specimens were embedded in Durcupan ACM (Fluka, Buchs, Switzerland), sectioned with an LKB Ultratome III with glass knives, picked up on Formvar-coated grids, and examined in an EM 300 Philips electron microscope.

RESULTS

Subcellular fractionation of extracts of Fusarium culmorum

To obtain membrane preparations, extracts were fractionated by differential and density-gradient centrifugation according to the following steps.

Step 1. Preparation of homogenate and cell-free extract. Cells were suspended in a medium containing 0.5 M-tris-HCl buffer (pH 7.2), 0.4 M-sucrose and 1 mM-EDTA. Twice the net weight of the cells in glass beads were then added. After 30 s of shaking in a Braun homogenizer, at least 80 % of the cells were broken. The homogenate was decanted and centrifuged at 3000 g for 10 min. The sediment was suspended with new portions of the suspension medium recentrifuged as above, in order to recover the membrane fragments which still adhered to the cell-wall material. The pellet was finally discarded and the supernatants of both centrifugations were mixed together, constituting the extract.

Step 2. Differential centrifugation. The extract was centrifuged at 40000 g for 30 min. The pellet, consisting of different types of membranous materials and mitochondria, was washed by resuspension by means of a Potter homogenizer and recentrifuged. Finally, the pellet was resuspended in a small portion of the suspension medium and fractionated by sucrose density-gradient centrifugation.

Step 3. Discontinuous sucrose density-gradient centrifugation. Attempts to fractionate the sediment of the 40000 g centrifugation by using a linear gradient resulted in poor resolution. For the fractionation of the 40000 g sediment, 4 ml of the suspended sediment were layered on top of a 60 ml capacity tube containing four layers of a discontinuous sucrose gradient consisting of 10 ml of 70 % (w/v) sucrose, 20 ml of 60 % sucrose, 15 ml of 55 % sucrose and 10 ml of 30 % (w/v) sucrose, all in 0.05 M-tris-HCl buffer (pH 7.2). The tubes were then centrifuged at 80000 g for 120 min in a Spinco L-265 B centrifuge.

The results of this centrifugation are shown in Fig. 1. After centrifugation, the brown material which constituted the 40000 g sediment was divided into four fractions which appeared as four distinct bands located on top of each one of the layers of the discontinuous gradient. The four bands were named fractions 70, 60, 55 and 30, corresponding to the percentage of sucrose in the layer immediately below as shown in Fig. 1.

Fig. 1. Fractionation of the 40000 g sediment on discontinuous sucrose gradients.

Table 1. *Specific activities of ATPase and Fumarase in the 40000 g sediment from extract of Fusarium culmorum and in the fractions resulting from its fractionation*

Fractions	ATPase	Fumarase	ATPase/fumarase ($\times 100$)
40000 g Sediment	0.66	7.2	9.1
Fraction 70	0.84	2.0	42.0
Fraction 60	1.4	5.3	26.0
Fraction 55	1.8	30.6	6.0
Fraction 30	0.33	6.0	6.0

Enzymic properties of the isolated fractions

In order to examine the nature of the isolated fractions, structural and enzymic criteria were used. The enzymic activity of ATPase is generally accepted to be present in the cytoplasmic membrane but it is also present in other organelles such as mitochondria. Therefore we decided to measure the activity of fumarase, a mitochondrial enzyme, as well as ATPase and to take the ratio of the two in each fraction as a measurement of the purity of the fraction. Table 1 shows the values for the specific activities of each of these enzymes in the different fractions as well as the ratio of the two. The maximum level of fumarase activity occurred in fraction 55 and was much lower in fractions 60, 70 and 30. The level of ATPase activity in the fractions did not differ very much except in fraction 30 where it was very low. The ratio of ATPase to fumarase was highest in fraction 70 and decreased along the gradient, being minimal for the fractions that were less dense. Fraction 70 had a high level of ATPase with a very low level of fumarase, and according to our criteria this must have been a fraction very rich in cytoplasmic membrane fragments and with a very low mitochondrial contamination.

Ultrastructure of the fractions

To examine the nature of the fractions we studied their ultrastructure. Fig. 2, 3 and 4 show the ultrastructure of fraction 70. It consists mainly of opened or closed triple-layered large membrane fragments (Fig. 4). These fragments were probably pieces of the cytoplasmic membrane which had broken during the homogenization process. The ultrastructure of this fraction again enabled us to distinguish it from fraction 55 (Fig. 5) which as expected consisted mainly of mitochondria. It was also distinguishable from fraction 30 (Fig. 6) which consisted of vesicles of different sizes, some of which had electron-dense contents.

Fig. 2. Electron micrograph of a thin section through fraction 70.
Fig. 3. Electron micrograph of a thin section through fraction 70.
Fig. 4. Electron micrograph of a thin section through fraction 70.
Fig. 5. Electron micrograph of a thin section through fraction 55.

Fig. 6. Electron micrograph of a thin section through fraction 30.

Chemical composition of fraction 70

Since according to our criteria fraction 70 must be rich in cytoplasmic membrane fragments, we examined its chemical composition. We found that it consisted of protein (25 % dry wt) and lipid (40 % dry wt) but also had a high carbohydrate content (30 % dry wt). Paper-chromatographic analysis of the carbohydrate showed only one spot which corresponded to glucose. These results are in agreement with those of Matile (1970) who obtained membrane preparations with 30 % carbohydrate content from *Saccharomyces cerevisiae*.

DISCUSSION

The fractionation of cell homogenates for the preparation of a particular type of cell component or organelle needs to be accompanied by methods to determine the distribution of the components or organelles in the different fractions. Since there is a loss of morphological information when a cell is broken, examination of the ultrastructure of the fractions is not enough to identify their components. The study of the enzymes present in a fraction has proved to be useful, especially when each enzyme studied is found in only one particular organelle. When the aim is the purification of cytoplasmic membranes, it is more difficult to interpret data from the study of enzyme activities. The only enzyme admittedly present in the cytoplasmic membrane is ATPase, but this activity is also found in mitochondria. Therefore we decided to study in our fractions the levels of ATPase and of fumarase, a typically mitochondrial enzyme, and to use the ratio of the two as a measurement of the purity of our membranes.

The method of fractionation that we have developed is the result of a series of attempts to find optimum conditions. We had observed that in 40000 g sediment there are mitochondria and different types of membranous materials, and that differential centrifugation at intermediate speeds does not permit the separation of these components. However, when this sediment was centrifuged in discontinuous sucrose gradients, it was divided into four fractions each then having a higher degree of homogeneity. Fraction 70 had the maximum value for the ratio ATPase/fumarase, and its ultrastructure showed that it contained membrane fragments having the typical 'triple-layered' structure. We can conclude that it must have consisted mainly of cytoplasmic membrane fragments. Fraction 60 also contained membrane fragments, but the proportion of mitochondria was higher. Fraction 55, because of its enzymic properties, must have been a mitochondrial fraction, the electron micrographs showing mostly mitochondria with typical cristae. Finally, fraction 30 consisted of vesicles and membrane fragments which were very different from those of fraction 70. Their enzymic properties were different and they were found in a zone in the gradient with a much lower density. They must have been related to the endoplasmic reticulum.

The fact that the cytoplasmic membrane fragments appeared in a zone of high density can be explained by their high carbohydrate content. This result is in agreement with that of Matile (1970) who obtained membranes with 30 % carbohydrate by fractionation of yeast cell homogenates. In membranes obtained from yeast protoplasts, on the other hand, the carbohydrate content was not higher than 6 % (García Mendoza & Villanueva, 1967; Longley *et al.* 1968) as was also found in membrane preparations we obtained from *Fusarium culmorum* protoplasts (C. Nombela and J. R. Villanueva, unpublished).

We can conclude that the method used for the preparation of membranes will much affect their carbohydrate content and that it is difficult to decide whether the carbohydrate is part of the cytoplasmic membrane or remains attached to it during the homogenization process. While it has been claimed that the carbohydrate content of the membrane could be digested by lytic enzyme attack, resulting in a low value for carbohydrate content, it also could happen that some of the polysaccharide materials in the wall or in the periplasmic space remain attached to the membrane during the homogenization process, resulting in a high value.

In order to determine accurately the composition of the cytoplasmic membrane, both the protoplast method of fractionation of homogenates as we have described and a more detailed qualitative analysis of the chemical components of both types of preparations

would have to be used. Such studies would contribute to the understanding of the problem of the structure and function of fungal membranes.

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