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A simple and rapid method for the purification of the mitochondrial porin from mammalian tissues

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A new, simple and rapid procedure for the purification of high amounts of mitochondrial porins from different tissues of mammalia is described. The method consists in a single step hydroxyapatite/celite chromatography of Triton X-100 solubilized mitochondrial membranes. For optimal purification several factors are critical such as the absence of salts, a low protein/detergent ratio and an exact hydroxyapatite/celite ratio of 2:1.

Porins are a class of proteins located in the outer membranes of mitochondria and of Gram-negative bacteria. In these membranes they form unspecific general diffusion pores with a diameter of approx. 1.3 nm in bacteria and approx. 1.7 nm in mitochondria, through which water-soluble molecules can easily permeate [1,2]. A great deal of information including amino acid sequences and X-ray diffraction studies is available on porins isolated from bacteria [1]. The progress on porins from mammalia has been hindered by the lack of a simple method for the purification of sufficient amounts of these proteins. First the porin from rat liver was purified starting from isolated outer mitochondrial membranes by a long and laborious procedure [3,4]. Later we have purified the pig heart and the rat brain porins from whole mitochondria. Also this procedure, however, is laborious since it includes three chromatographic steps, besides solubilization of mitochondria [5,6].

In this paper we report a very simple and rapid procedure for the purification of mammalian porins with high yield. The method consists of a single step hydroxyapatite/celite chromatography of Triton X-100 solubilized mitochondria and leads to the isolation of pure porin.

Materials. Hydroxyapatite (Bio-Gel HTP) was purchased from Bio-Rad. The batch of hydroxyapatite is not critical for optimal purification of porin in difference to the experience with other transport proteins [7,8]. Hydroxyapatite, which was prepared according to Tiselius [9], was also used. Celite was obtained from Roth (Celite 535) or from Serva (Celite 545).

Methods. Mitochondria from different tissues of mammalia were lysed by osmotic shock in 10 mM Tris-HCl and 1 mM EDTA (pH 7.0). After centrifugation, the pellet mainly consisting of mitochondrial membranes was solubilized by 3%. Triton X-100, 10 mM Tris-HCl (pH 7.0) and 1 mM EDTA at a final concentration of 5 mg protein/ml. This protein concentration was chosen in order to dilute the endogenous cardiolipin, since this phospholipid was shown to prevent the elution of porin from hydroxyapatite [5,7] and, vice versa, to induce the elution of other proteins

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[7]. In fact, it was found that, on increasing the protein concentration in the solubilization medium above 5 mg/ml, both the absolute and the relative amount of porin in the final preparation progressively decreases. After 30 min at 0 °C, the solubilization mixture was centrifuged at 18000 rpm for 30 min. The supernatant was applied on a dry, well packed hydroxyapatite/celite (6 g, ratio 2 : 1) column (1.3 × 10 cm). Elution was performed with the solubilization buffer. Porin was the only protein eluted under these conditions. The first 12 ml were usually collected. The same results were obtained by using preequilibrated columns. In this case it was convenient to mix hydroxyapatite/celite volumes (2 : 1) calculated from sedimented materials. Protein determination, SDS-polyacrylamide gel electrophoresis and staining were performed as previously described [5]. Peptide mapping was obtained by *Staphylococcus aureus* proteinase V8 proteolysis carried out on slices of SDS gel according to Cleveland et al. [10].

Results and discussion. It is known that relatively few proteins are eluted from hydroxyapatite columns when Triton-solubilized mitochondria are applied [5,7]. Also celite (diatomaceus earth or Kieselgur) has successfully been used for the purification of some very hydrophobic proteins [11]. The presence and the relative amount of proteins in the pass-through, however, greatly depend on the composition of the medium in which the proteins are solubilized and applied to dry hydroxyapatite and celite columns [7,11]. Fig. 1 shows the effect of increasing concentrations of KCl on the polypeptide pattern of the eluate obtained after hydroxyapatite/celite (1 : 1) chromatography of Triton X-100 solubilized pig heart mitochondria. When no KCl is present in the solubilization medium (lane 0), the hydroxyapatite/celite eluate contains a considerable amount of porin corresponding to the band of apparent molecular weight of 35 kDa. Under these conditions porin is already largely enriched with only one minor contaminant. Increasing the concentrations of KCl in the solubilization medium causes a progressive increase in the number and in the total amount of proteins which are eluted from the column. The degree of purification with respect to porin was negatively affected by any other salt tested, even at low concentrations (10–20 mM). Thus, in contrast to the majority of proteins, porin is eluted from hydroxyapatite and celite without the need of salt. indicating only minor or no interactions between this protein and the adsorption materials used.

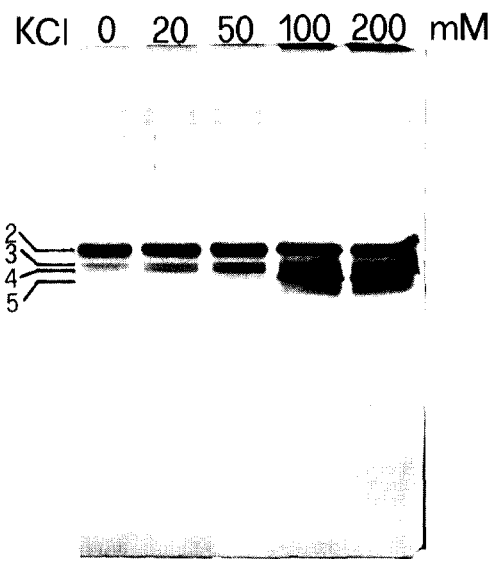

Fig. 1. Influence of increasing concentrations of KCl in the solubilization medium on the purification of porin by hydroxyapatite/celite (1 : 1) chromatography. Mitochondrial membranes from pig heart were solubilized in 3% Triton X-100 (w/v), 20 mM KH_2PO_4 (pH 6.5) and 1 mM EDTA at 10 mg protein/ml in the presence of no KCl (0), 20 mM KCl (20), 50 mM KCl (50), 100 mM KCl (100) or 200 mM KCl (200). After centrifugation at 138000 × *g* for 30 min, 0.6 ml of supernatants were applied on hydroxyapatite/celite (1 : 1) columns (pasteur pipettes containing 0.6 g of dry materials). The first 0.6 ml eluted were collected and analyzed. Numbers 2–5 identify mitochondrial membrane proteins as follows: band 2, porin; band 3, the phosphate carrier; band 4, the 2-oxoglutarate carrier; band 5, the ADP/ATP carrier (see Refs. 5, 7 and 11).

In order to optimize the experimental conditions, we have investigated the effect of different ratios of hydroxyapatite and celite on the purification of porin. The analysis by SDS gel electrophoresis of the eluates from columns with hydroxyapatite/celite ratios between 9 : 1 to 1 : 9 is shown in Fig. 2A. The control with hydroxyapatite alone (first lane on the left) shows the normal electrophoretic pattern of the first portion of the hydroxyapatite pass through, characterized by an excess of adenine nucleotide carrier and porin and by the presence of phosphate carrier

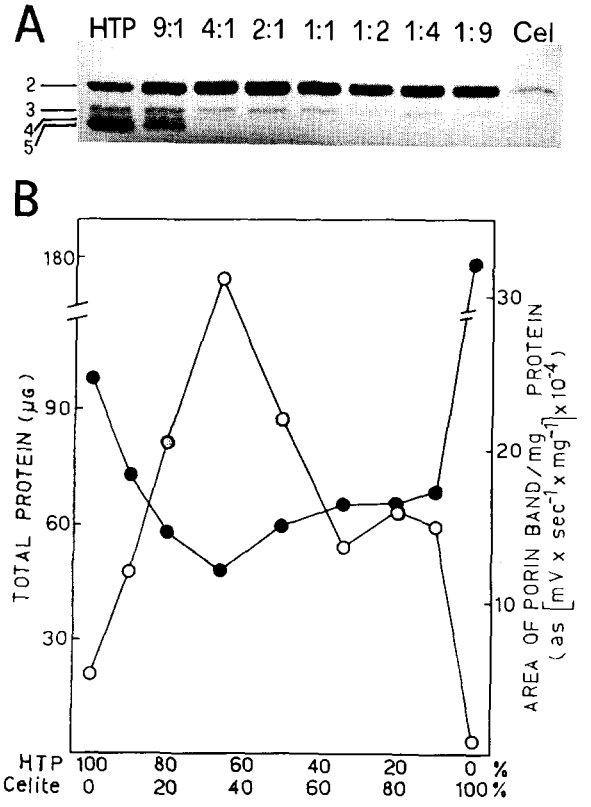

Fig. 2. (A) SDS gel electrophoresis of the eluates obtained from columns containing different ratios of hydroxyapatite and celite. Only the portion of the slab gel corresponding to polypeptides of molecular mass in the range 30–36 kDa is shown. There are no polypeptides in the rest of the gel except where the eluate from celite alone was analyzed (lane Cel). Numbers 2–5 identify mitochondrial proteins as follows: band 2, porin; band 3, the phosphate carrier; band 4, the 2-oxoglutarate carrier; band 5, the ADP/ATP carrier (see Refs. 5, 7 and 11). (B) Dependence of the total protein (●) and the relative amount of porin (○), present in the eluates, on the ratio between hydroxyapatite and celite. Conditions as in Fig. 1. The relative amount of porin is expressed as area of porin/mg protein. The area of porin was calculated from densitometric traces of SDS gels by means of a LKB 2202 Ultroscan Laser Densitometer coupled to a Shimadzu C-R 1B.

and 2-oxoglutarate carrier [5,7,11]. The addition of celite to hydroxyapatite progressively decreases the elution of the substrate carriers with respect to porin. Also the absolute amount of porin increases on adding celite to hydroxyapatite until a hydroxyapatite/celite ratio of 2:1. In Fig. 2B the protein content and the relative amount of porin in the eluates are reported as a function of hydroxyapatite/celite ratios. With hydroxyapatite alone or celite alone a relatively large amount of protein passes through the column. Any combination of hydroxyapatite and celite decreases the elution of protein with a minimum at a hydroxyapatite/celite ratio of 2:1. In contrast to this the relative amount of porin, as calculated from densitometric profiles, shows a maximum at the same 2:1 ration. It should be noted that in both the experiments of Fig. 1 and Fig. 2 optimal conditions were not entirely fulfilled (see legends to these figures). Under the optimal conditions described in the Methods section the procedure leads to a completely pure preparation (Fig. 3). This figure also shows that the method can successfully be applied to the purification of porin from different tissues. Thus, the porins isolated from pig heart, from heart, brain, kidney and liver of rat (Fig. 3) or of other mammalia (not shown) all consist of a single protein band when analyzed by SDS gel electrophoresis. They have a very similar electrophoretic mobility corresponding to an apparent molecular mass of 35.5 kDa, with the exception of porin from pig heart which has a slightly lower molecular mass (35 kDa). The yield of the purified protein varies from 0.5 to 1.5% of the total mitochondrial protein in relation to the tis-

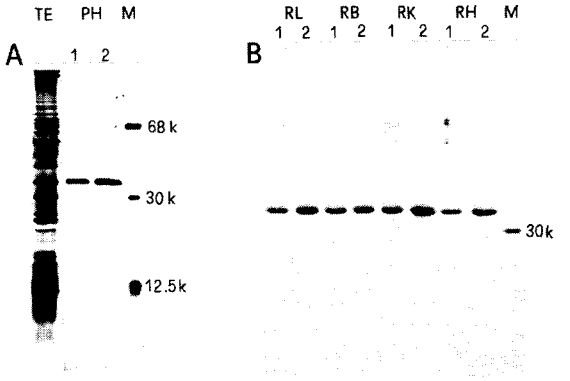

Fig. 3. SDS gel electrophoresis of mammalian porins purified by one step hydroxyapatite/celite chromatography. Conditions as described in Methods. (A) 17.5% acrylamide (acrylamide/bisacrylamide ratio 30:0.2); (B) 12.5% acrylamide (acrylamide/bisacrylamide 30:0.8). 10 µg (in 1) and 20 µg (in 2) of purified porin from pig heart (PH), rat liver (RL), rat brain (RB), rat kidney (RK) and rat heart (RH) were applied to the gel. TE, Triton X-100 extract of pig heart mitochondria; M, markers (bovine serum albumin, 68000; carbonic anhydrase, 30000 and cytochrome *c*, 12500).

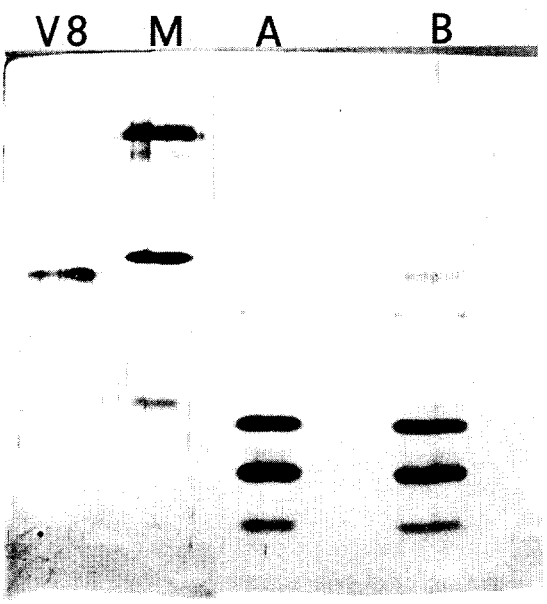

Fig. 4. Comparison of peptide maps of pig heart porin purified with the present and the previous method. Gel slices containing about 3 μg of pig heart porin were re-run in the presence of 0.3 μg *S. aureus* proteinase V8 as described by Cleveland et al. [10]. V8, 1 μg of *S. aureus* proteinase V8; M, markers as in Fig. 3; A, porin purified as in Ref. 5; B, porin purified as described in Methods.

sue used for the purification. The highest values were obtained with kidney and the lowest with liver. It is interesting that the amount of purified porin from rat-liver mitochondria closely corresponds to the porin content of these mitochondria as deduced by titration with specific antibodies [12]. In any case the yield of porins purified with the present procedure is at least two-fold higher than that obtained by the previous methods [3–6].

All the mammalian porins isolated by the procedure presented in this paper show the same functional properties as compared to the porins purified as previously described [4–6]. Thus, (a) their incorporation into artificial bilayer membranes results in a stepwise increase of the membrane conductance, due to the opening of pores; (b) their single-channel conductance is about 4 nS in 1 M KCl, corresponding to an average pore diameter of 1.6–1.7 nm; (c) the size of the pores is voltage dependent since the single-channel conductance switches to substates on increasing the voltage; and (d) the pores are slightly anion selective. A further proof of the identity of the mammalian porins purified by the present method with those purified with the previous procedure [5] is given by the comparison of the respective peptide patterns after digestion with *S. aureus* proteinase V8 (Fig. 4). When mammalian porins are degraded by *S. aureus* proteinase, a typical peptide pattern is obtained which is characterized by the presence of only three peptides with an apparent molecular mass of 15, 10 and 7 kDa [13]. The results obtained with the porins isolated by the use of the method described in this paper are virtually indistinguishable from those observed with the porins purified previously (cf. lanes B and A of Fig. 4).

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