PRO EXPERIMENTIS

Crossed Immunoelectrophoresis: An Alternative Method for Identification, Quantification and Characterization of Membrane Proteins

Analysis of membrane proteins by crossed immunoelectrophoresis[1] is described. The method possesses a high resolving power[2] and allows enzymatic characterization[3]; but a proper analysis of membrane components has been hindered by denaturation or incomplete solubilization together with unspecific precipitation in the gel[4–6]. In the presence of a solubilizing agent, obstruction of the immunoreaction may also occur. However, non-ionic detergents, with which a high degree of solubilization of membrane proteins is possible, do not prevent the antigen-antibody reaction or greatly affect the activity of enzymes[1a, 3, 7, 8].

Human erythrocyte membranes[9] were solubilized by repeated treatment with a solution containing 0.0038 *M Tris* and 0.01 *M* glycine (pH 9.2, 5 °C) and 1% Berol EMU-043. The degree of solubilization obtained with this and other non-ionic detergents (Triton X-100, Lubrol WX, Nonidet P 40) has been found to be 95–99%[10]. With Berol the same results were found for membrane proteins of ox, pig, dog, cat, rat, and rabbit erythrocytes. The proteins of bovine milk fat globule membranes, isolated by centrifugation from butter milk and butter serum[11] (obtained after churning a sample of washed cream from pooled milk[12]) were solubilized in Berol by repeated treatment and sonication to an extent of 50%.

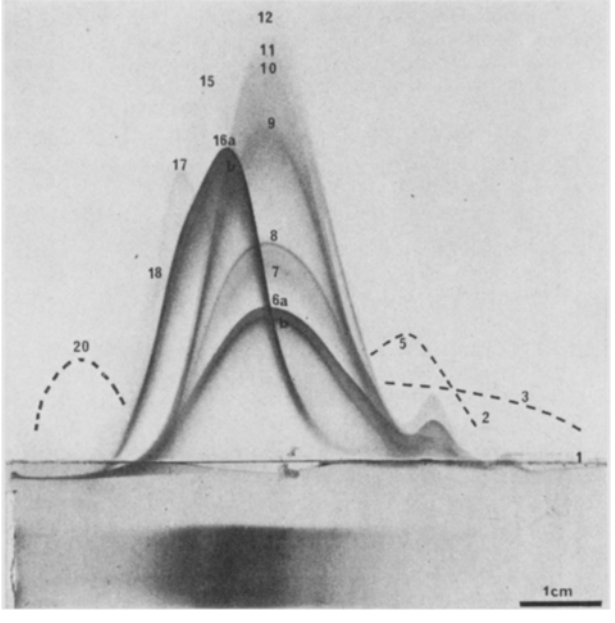

Fig. 1. Crossed immunoelectrophoresis of human erythrocyte material at pH 8.9 (16 °C) (*Tris* 0.038 *M*, glycine 0.01 *M*) in 1% agarose gels (Litex, Batch 060, Glostrup, Denmark) containing 1% (w/v) Berol EMU-043 (MoDoKemi, Stennungsund, Sweden). A 10 µl sample containing 36 µg of solubilized membrane protein was separated by applying 10 V/cm for 70 min (anode to the right). Subsequently the antigens were subjected to electrophoresis through an antibody-containing gel with 41 µg/cm² by applying 3 V/cm for 16 h (anode at top). The fixed and Coomassie Brilliant Blue stained proteins of the first dimension electrophoresis are inserted by photomontage. 16 different precipitation lines were seen on the original plate. Precipitate No. 1 represented a split product of No. 16. No. 2 was identified as albumin by intermediate gel technique[1c]. Precipitates Nos. 6–12 correspond to Spectrin[16]. Acetylcholine esterase activity was found where line No. 20 is inserted[8].

Crossed Immunoelectrophoresis according to CLARKE and FREEMAN[2] of Berol-solubilized human erythrocyte membrane proteins, freshly prepared or stored frozen (−20 °C), demonstrated up to 20 precipitates when the detergent was incorporated in the gel (Figure 1). Examination of 12 membrane preparations revealed that the pattern of precipitation arcs was reproducible when the same batch of antibodies was used[10]. Each precipitate was identified by 1. its electrophoretic migration relative to that of albumin (Figure 1), 2. its staining intensity and 3. its individual shape. Thus, a number could be assigned to each precipitate and the pattern used as a reference (Figure 1). The antibodies were raised in rabbits[1a, 10] and caused precipitation of all solubilized protein. Due to interaction between the solubilized material and some serum proteins[13], it was necessary to purify the antibodies[1d].

The immunoelectrophoretic method is quantitative, as the area delimited by a precipitation arc is proportional to the applied amount of antigen[2]. The method is also very sensitive: as little as 0.1 µg of erythrocyte membrane proteins gave well-defined precipitates after Coomassie Brilliant Blue staining. Using samples stored frozen, the day-to-day reproducibility of the method, as measured by variation of the areas outlined by the precipitation arcs, was nearly as good as that obtained by using serum proteins[1b, 10].

Applying the same technique, the solubilized protein of milk fat globule membranes revealed a precipitation of about 13 distinguishable precipitates (Figure 2). The precipitates were not so well defined as for erythrocyte membrane proteins, but the pattern was concordant for 4 different preparations.

The membrane-specific origin of the precipitates was established by use of antibodies against human serum and bovine whey (Dakopatts A/S, Copenhagen) (Figures 1 and 2)[1c].

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For the two membrane systems under investigation, the resolving power of crossed immunoelectrophoresis is similar to that of the widely-used SDS polyacrylamide gel electrophoresis, the former method giving 20 and 13 precipitates for the two systems, respectively, in comparison with 16 and 12 major bands when using the latter[14,15]. However, the separation obtained with the two methods differs, as it is based on different molecular properties. This has been clearly demonstrated for the erythrocyte membrane protein Spectrin, which appears as two bands in SDS-gel electrophoresis but reveals at least 7 precipitates by quantitative immunoelectrophoresis (Figure 1)[16]. Thus, these methods supplement each other; and immunochemical examination of membrane proteins separated by preparative SDS-gel electrophoresis is in progress.

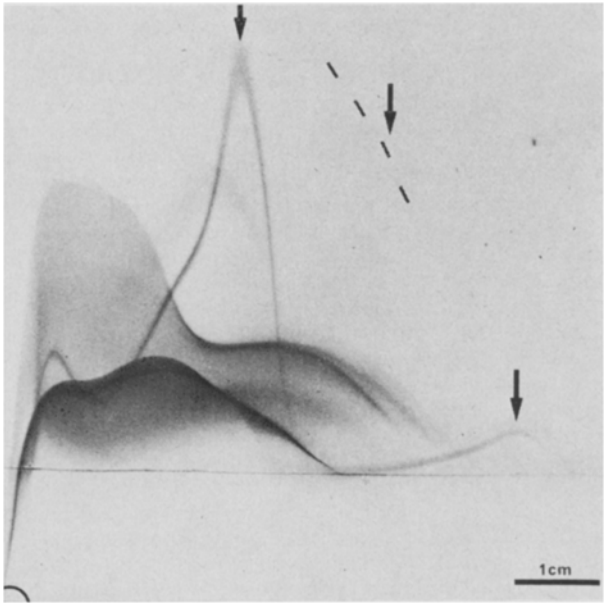

Fig. 2. Crossed immunoelectrophoresis of a 100 µl sample containing 145 µg Berol EMU-043 solubilized membrane proteins from bovine milk fat globules. The antibody content of the gel was 10 µg/cm². 11 different precipitates were seen on the original plate. The precipitates indicated with arrows were also present in bovine whey[1c] in very low concentrations. The conditions were as given in the legend to Figure 1, except for the agarose used (Batch 091 with lower electroendosmosis).

Zusammenfassung. Das Ergebnis der Antigen-Antikörper-Kreuzelektrophorese von Membranproteinen aus Erythrocyten und Fettkügelchenhüllen, solubilisiert mit nicht-ionischen Detergenten, wird beschrieben.

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Organogenesis in Callus Culture of *Gazania splendens* MOORE Induced on New Medium

In vitro cultures of plants make it possible to obtain material of great breeding value (haploids, diploid isogenic lines, polyploids, gene mutations, parasexual hybrids, etc.)[1,2]. This type of culture has not yet been used in the tribe Arctotideae (Asteraceae)[3–5]. However, the induction of organogenesis is the basic requirement for the application of the in vitro culture in this tribe.

Mature leaves of the starting plant (Figure 1) *Gazania splendens* MOORE (Sempra, Prague) were used as primocultures. Leaves sterilized on the surface were cut vertically to the petiole to sections of 0.25 cm², and placed by the reverse side on a medium (Table) according to MURASHIGE and SKOOG[6]. The cultures were placed in a thermostat in the dark at 25 °C.

In about 20 days the cultivated pieces of the leaves created in the centre calli which grew, and 57–76 days after establishing the primocultures they could be passaged. Upon passage on the same medium the calli grew quickly and within 14 days attained an average fresh weight of 2 g. They were consistent and nodulous, their basic colour in the R.H.C. system[7] was 160 D. They showed no signs of organogenesis, even when 2,4-D was removed from the medium (Figure 2).

When the calli were passaged on a new medium designated NNS (Table) and placed in a thermostat in the light (25 °C, approx. 7000 lux), organogenesis took place rapidly. In 7 days white roots appeared on the calli growing heliotropically. After 14 days a branched root system growing geotropically was formed which after 10 days expanded into the whole medium. At the same time green buds appeared massively on the calli. After transferring the buds with a small piece of callus and a few small roots again on medium NNS, dark green leaves developed within 4 days to 2 weeks, corresponding morphologically to the young leaves of *Gazania splendens* MOORE. The calli from the 5th passage on the medium according to MURASHIGE and SKOOG also retained fully

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