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A METHOD FOR DETERMINING THE N-TERMINAL RESIDUE OF PROTEINS FIXED IN POLYACRYLAMIDE GELS

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SUMMARY

A quick and convenient method has been devised for determining the N-terminal residue of a protein fixed in polyacrylamide gels. The method is a combination of isoelectric focusing, eventually replaced by disc electrophoresis, dansylation of protein without eluting from polyacrylamide gel, and identification of 1-dimethylaminonaphthalene-5-sulphonyl amino acids, after hydrolysis, by two-dimensional thin-layer chromatography. It can be applied to an amount of protein of the order of 200 μg, incompletely purified, or to a mixture of proteins, when a suitable system of separation by electrofocusing is available.

INTRODUCTION

In our sequence studies, we are often faced with the problem of determining the N-terminal residue of a protein which appears to be chromatographically pure but which gives several bands when it is submitted to electrophoresis or electrofocusing in polyacrylamide gels. If performed directly on such material, N-terminal amino-acid determination can give ambiguous results, especially if the protein has two N-terminal residues, which is the case in several of the trypsinogens we have studied.

We have devised a method which enables us to check the purity of our protein material and at the same time to determine its N-terminal residue. The protein is first submitted to electrofocusing on polyacrylamide gel by the method of WRIGLEY[1] or to disc electrophoresis in a suitable system. The protein bands are located either by comparison with a stained gel, or by precipitation in trichloroacetic acid if enough material is available. Corresponding bands from several gels are sliced and pooled. Dansylation is performed without elution and 1-dimethylaminonaphthalene-5-sulphonyl (dansyl) amino acids are identified after hydrolysis by thin-layer chromatography on polyamide[2].

This method has the advantage over previously described methods, involving the dinitrophenyl derivatives[3] or the phenylthiohydantoins[4], being much more sensitive and much less time-consuming.

MATERIALS AND METHODS

Isoelectric focusing

Essentially, the photopolymerization method of WRIGLEY[1] was used throughout this study. Acrylamide, bis-acrylamide, ampholine mixture (LKB Produkter AB, Stockholm-Bromma 1, Sweden) protein sample and riboflavine are mixed together, poured into columns of dimensions 0.5 × 11 cm and then photopolymerized. The gel columns are placed in the electrophoresis tank with 0.2 % sulphuric acid in the anodic compartment and 1 % sodium hydroxide in the cathodic compartment. A current of 2 mA/gel is run through the gels. After about 0.5 h, the potential reaches 300 V; the instrument is then set to constant voltage and the intensity subsequently decreases. The electrophoresis is stopped after 4 h.

Staining and slicing the gels

All the extruded gels but one are immersed in 10 % trichloroacetic acid. The amount of protein necessary to produce white precipitation bands depends on the nature of the protein; the last gel is always stained with bromophenol blue, according to AWDEH[5], without removing the carrier ampholites. The corresponding bands from several gels are sliced with a razor blade, pooled, suspended in water in a dialysis bag, crushed between the fingers, dialyzed against water and lyophilyzed.

N-Terminal amino-acid determination

Dansylation is performed by the method of GROS AND LABOUESSE[6] on the lyophilyzed material, in the presence of acrylamide. The latter does not interfere with the procedure and is removed at a later stage. The conditions used for dansylation and hydrolysis are those described by GROS AND LABOUESSE[6] for proteins, except that a delay of 2 h is introduced after precipitation in trichloroacetic acid in order to facilitate sedimentation of acrylamide. Also, before hydrolysis in 1 ml of 6*N* HCl, the precipitate is lyophilyzed to avoid excessive dilution of the acid used for hydrolysis.

The hydrolysate is cooled in an ice bath which causes the acrylamide to precipitate. The liquid is pipetted off, evaporated to dryness, dissolved in 1 ml of water, and extracted with 3 × 1 ml of ether. The water and the ether phases are evaporated and an aliquot of each phase is used for the identification of dansyl amino acids by thin-layer chromatography.

Thin-layer chromatography of dansyl amino acids by the method of Woods and Wang[2]

The polyamide layers are from Chen Chin Trading Co, Taipei, Taiwan (polyamide sheets from other origins have been tried without success). The 15 × 15 cm sheets are cut into four smaller sheets of 6 × 6 cm, not only for economy but also to save time. In fact, a complete run in the two solvents takes only about 40 min. The evaporated ether or aqueous phase is dissolved in 20 μl of acetone–acetic acid (2:1), and a very small drop is deposited with a pointless syringe needle (Benton, Dickinson, France) in the corner of the 6 × 6 cm polyamide sheet, making a spot of diameter not greater than 1 mm, at 0.5 cm from each edge. The sheet is put into a small jar containing a layer of water–98 % formic acid (100:1.35). When the solvent reaches the upper edge, the sheet is removed, dried with a hair dryer for a few minutes, then put in another jar containing benzene–acetic acid (9:1), at a right-angle to the

first direction. When the solvent reaches the upper edge, the sheet is dried and visualized in UV light. A Chromato-Vue (Ultra-Violet Products Inc., San Gabriel, Calif., U.S.A.) is very convenient for this purpose. The fluorescent spots are marked with a pencil, since they become fainter after a few days. The plates, illuminated in UV light, can also be photographed with a colour film through the special filter of the Chromato-Vue. The dansyl amino-acid spots are very easily identified by comparison with a test mixture containing about 10 ng of each dansyl amino acid run in the same solvents.

RESULTS AND DISCUSSION

Figs. 1 and 2 illustrate the results obtained by applying this method to sheep S-sulphotrypsinogen. Sheep trypsinogen has two N-terminal residues, phenylalanine and valine[7]. When activated, it liberates two N-terminal peptides, a hexapeptide

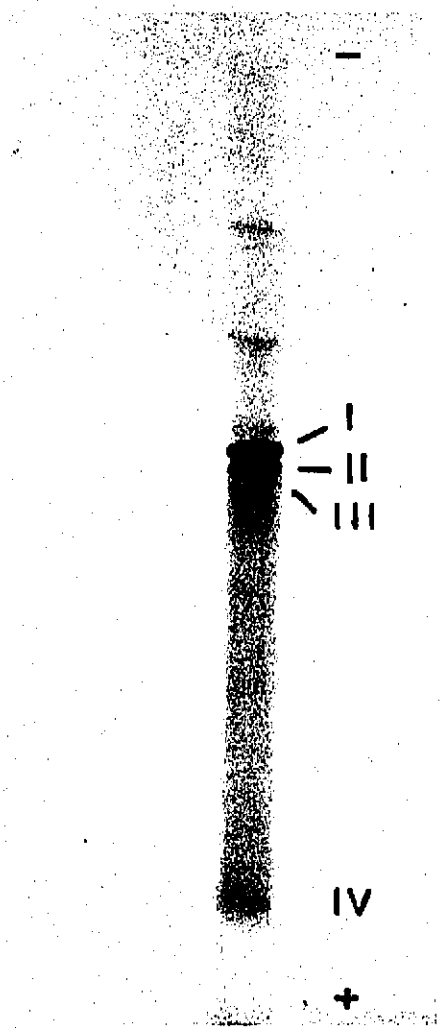

Fig. 1. Electrofocusing of 750 μg of sheep S-sulphotrypsinogen in a 10% polyacrylamide gel containing carrier ampholites of pH 3–5, photopolymerized with riboflavine in a column of dimensions 0.5 × 11 cm. Duration of electrofocusing, 4 h at 300 V. Gel stained with bromophenol blue.

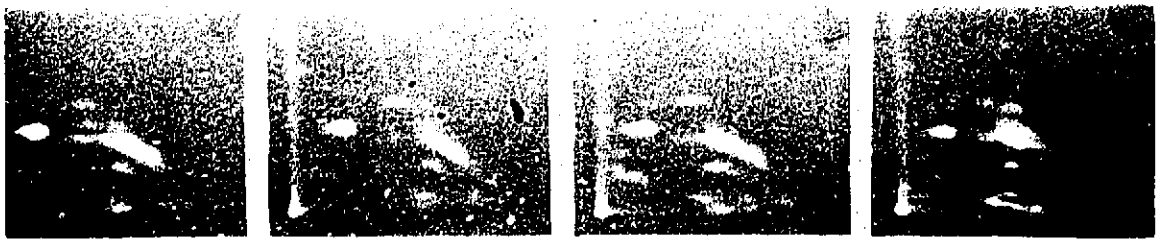

Fig. 2. Top: thin-layer chromatography of a test mixture of dansyl amino acids on polyamide (about 10 ng of each). Bottom: thin-layer chromatography of dansyl amino acids obtained by dansylation of proteins precipitated in gels by trichloroacetic acid after electrofocusing. The bands I, II, III and IV of Fig. 1, from left to right, respectively, have been sliced. First dimension: water 08%, formic acid (100:1.35) (from left to right). Second dimension: benzene acetic acid (9:1) (from bottom to top).

Val-Asp-Asp-Asp-Asp-Lys and an octapeptide Phe-Pro-Val-Asp-Asp-Asp-Asp-Lys. We have concluded that sheep trypsinogen is a mixture of two molecules, one beginning with the sequence of the hexapeptide and the other beginning with the sequence of the octapeptide, without presuming any additional differences which may exist along the chains. Any attempt to separate these two trypsinogens has failed, however.

Sulphitolysis is reported by PECHÈRE *et al.*[8] as a mild treatment to break the disulphide bridges and to oxidize cystinyl groups to S-sulphocysteyl groups without altering other residues. Sheep trypsinogen has been sulphitolysed by the method of PECHÈRE *et al.*[8] and 3 mg of S-sulphotrypsinogen have been distributed and electrofocused in four gels, using the carrier ampholites at pH 3–5. One of the gels, stained with bromophenol blue (Fig. 1), shows several bands of which four also appear in the trichloroacetic acid-fixed gels. The corresponding bands, marked I, II, III and IV, have been sliced from the trichloroacetic acid-fixed gels, pooled and dansylated. Dansyl amino acids found in the ether extract of the hydrolysate are easily identified (Fig. 2) by comparison with the test sample (Fig. 3); phenylalanine is the main N-terminal residue, valine being the next important residue. Since only N-terminal arginine and histidine are to be expected in the aqueous phase[6], this phase is usually left aside for an eventual control. It can be concluded from these results that the four bands are derived from S-sulphotrypsinogen and strictly speaking are not impurities. Sheep S-sulphotrypsinogen is not as homogeneous as that reported for the bovine enzyme. Sheep trypsinogen itself, when submitted to electrofocusing at pH 7–10, gives one major and one minor band. By the above procedure, phenylalanine and valine can be easily identified as N-terminal residues of the two bands (Fig. 4). In particular, no isoleucine is detected, which means that activation into trypsin does not occur during electrophoresis.

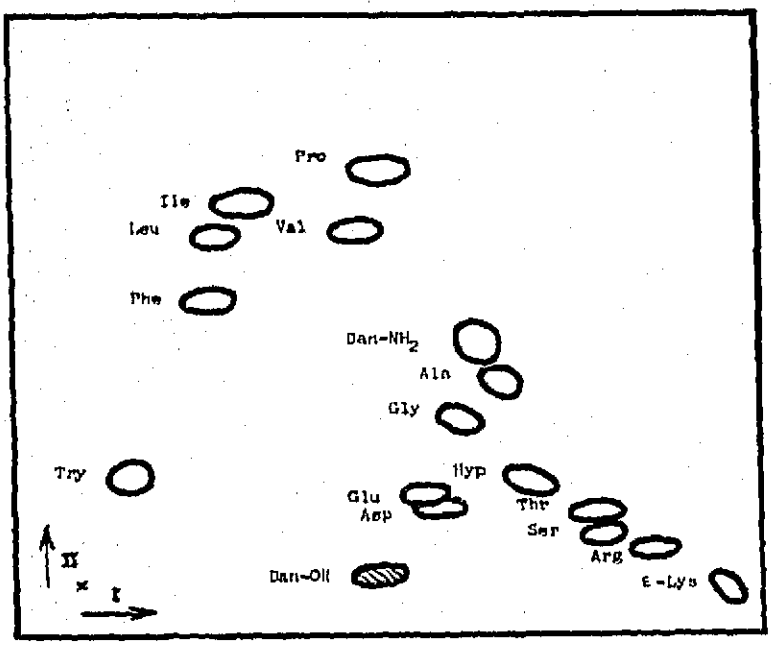

Fig. 3. Identification of spots produced by a test mixture of dansyl amino acids chromatographed on a thin layer of polyamide using the conditions described in Fig. 2 (about 10 ng of each). Dan = 1-dimethylaminonaphtalene-5-sulphonyl.

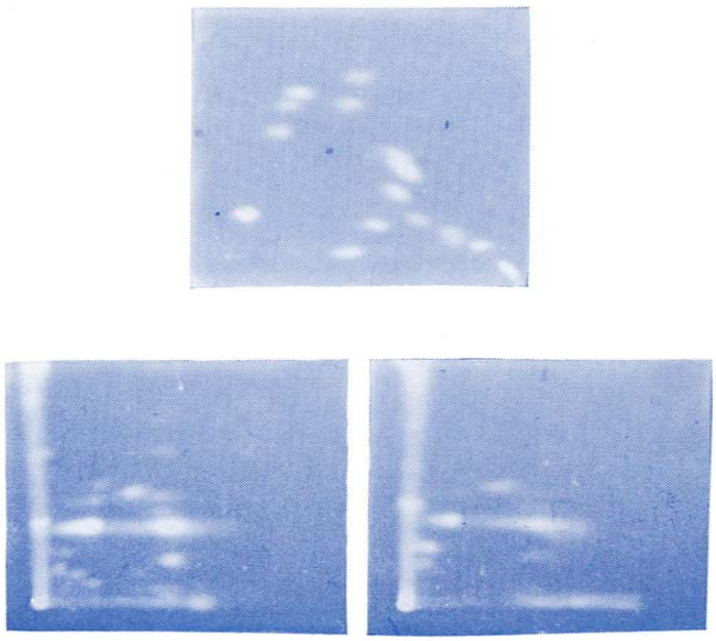

Fig. 4. Top: thin-layer chromatography of a test mixture of dansyl amino acids. Bottom: thin-layer chromatography of dansyl amino acids obtained by dansylation of the two bands of sheep trypsinogen after electrofocusing at pH 7–10. The conditions are the same as in Fig. 2.

S-Sulphotrypsinogen has been chosen as an example but the method is of general use. It can be applied to bands obtained by conventional electrophoresis and to incompletely purified proteins of which only a small amount is available. The minimum amount of protein depends on each particular case. In our experience, it is of the order of 200 μg. Theoretically, the method could be applied to much smaller quantities, since only a small aliquot of the hydrolysate is used for identification. One must bear in mind, however, that after dansylation, all dansyl amino-acid spots always appear on the polyamide sheet as a faint background. The relative importance of the background and of the N-terminal dansyl amino acid must be such that the latter is at least several times more intense than the background spots. In fact, this background helps to identify the spots and renders the chromatography of the test mixture unnecessary.

The time of hydrolysis is important. GROS AND LABOUESSE[6] recommend 18 h hydrolysis when dansyl valine or dansyl isoleucine are involved. In practice, we make two separate hydrolyses, during 4 and 18 h, respectively, and run the two samples separately. When trypsinogen is involved, the dansyl valine spot is much more intense after 18 h hydrolysis, while dansyl phenylalanine is almost the same in both hydrolysates. In cases when dansyl proline is present, the 4 h hydrolysate is essential, since dansyl proline is almost completely destroyed after 18 h.

In our hands, the whole procedure takes 2–3 days. The time-limiting step is elimination of trichloroacetic acid and carrier ampholites by dialysis. Two-dimensional chromatography on a thin layer of polyamide, performed on very small sheets, is particularly quick and convenient. The complete run in the two solvents takes about 40 min. A necessary condition is to make a very small spot (diameter about 1 mm) and not to overload the layer: an amount of about 10 ng of dansyl amino acid is suitable. Dansyl hydroxide and dansylamide do not interfere with the chromatographic procedure and appear as distinct spots.

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