Peptide mapping of polypeptides separated by two-dimensional electrophoresis: Protease digestion directly on the two-dimensional gel followed by electrophoresis in reverse direction

A method is described which allows to reveal simultaneously the proteolytic patterns of numerous polypeptides separated by two-dimensional electrophoresis. After two-dimensional electrophoresis, the gels were dipped successively in buffers for preequilibration, protease digestion, and reequilibration. They were then returned to the electrophoresis tank, and electrophoresis was continued for a short time. After silver staining, digestion products appeared, lined up behind the original polypeptide spots. The method allows proteolytic patterns of numerous polypeptides to be visualized simply and quickly. Among proteins of wheat leaves, 31 groups of related polypeptides were found according to the similarity of their proteolytic patterns, which are based on elution of proteins from 2D gels prior to digestion. We have developed a technique that allows the observation of the proteolytic patterns directly on the 2D gel.

1 Introduction

Some of the spots revealed by two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) of complex biological samples can correspond to related polypeptides: for example, two spots can correspond to the products of allelic genes or of homoeoallelic genes if the studied species is a polyploid, or to the products of a single gene differing by post-translational modifications [21]. For the interpretation of experiments using 2D-PAGE, knowledge of the relations between the individual polypeptides can be useful. Structural relations between several polypeptides may be detected by comparing patterns following proteolytic digestion. However, it would be too time-consuming to do it for numerous polypeptides with the techniques currently available [3–5].

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Abbreviations: 2D, two-dimensional; IEF, isoelectric focusing; PAGE, polyacrylamide gel electrophoresis; SDS, sodium dodecyl sulfate
2.3 % sodium dodecyl sulfate (SDS), 10 % sucrose. The second-dimensional separation was performed on 215 x 240 x 1 mm slab gels backed to GelBond PAG film. Several gels (11 % T, 2.65 % C, 0.5 M Tris-HCl, pH 8.8, 0.15 % SDS, 1 % sucrose) were simultaneously run in a DALT tank for 20 gels [8], and simultaneously silver stained in the apparatus described in [9]. The silver staining method was performed according to [10], with the modifications described in [11].

2.3 Proteolysis method

The “standard” procedure will be described below and any modifications will refer to this one. All steps of equilibration were done under gentle agitation, with 500 mL per gel. The procedure was as follows: 1) 2D-PAGE was performed as described in Section 2.2. 2) Gel equilibration for proteolysis: the 2D gels were removed from the apparatus and dipped in a 0.2 M Tris-HCl, pH 7, buffer for 20 min (two baths of 10 min). 3) Proteolysis: the gels were transferred to another bath of the same buffer containing additionally 0.18 mM (50 µg/100 mL) ficin (Sigma Product No. F 6008) for 30 min. The temperature was adjusted to 20 °C before incubation. 4) Gel equilibration for electrophoresis: the gels were dipped in the “tank buffer” (25 mM Tris, 0.2 M glycine, 0.1 % SDS) for 15 min (first bath for 5 min and second bath for 10 min). 5) Continued electrophoresis: the gels were returned to the apparatus. Clamps were used to prevent inclusion of air bubbles or tank buffer. The gels were positioned with the top oriented downwards and electrophoresis restarted in the opposite direction. The presence of GelBond made the gel handling easier, and was helpful for positioning them correctly. Electrophoresis was performed for 1 h at a constant current of 0.7 A.

3 Results and discussion

3.1 Restart of migration and protease activity

The best buffer conditions for protease activity and for the restart of migration were different. Preliminary experiments showed that the tank buffer was optimal for restarting migration. Figs. 1a and 1b show a gel run under normal conditions and a control gel (standard method without protease at step 3).
No vertical streaking was observed on the latter, which shows that the restart of the migration was good. By contrast, if migration was restarted in the same direction as before protease treatment an additional front appeared (see Fig. 3) proving that equilibration step 4 was not adequate for complete reequilibration with tank buffer. In a number of experiments we have tried to find a buffer allowing good ficin activity and compatibility with good migration, restarting electrophoresis. Optimal conditions for ficin activity are pH 7 and 37 °C, but already 20 °C proved adequate. A high molarity of Tris buffer, pH 7, was necessary because at low molarity (25 mM), migration on restarting electrophoresis was inferior despite equilibration step 4. Other buffers were also tested and, as expected, replacing the buffer of steps 2 and 3 by the tank buffer resulted in a good restart of migration (without an additional front), but ficin activity was lowered considerably. Incubation in the tank buffer, adjusted to pH 7 with HCl, resulted in a relatively good restart of migration, but ficin activity was still reduced, although less than with regular tank buffer. The tank buffer without SDS, either adjusted to pH 7 or nonadjusted, resulted in a bad restart of migration. Best results were obtained with the two steps of equilibration (steps 2 and 4). If either step 2 or step 4 were omitted the results were acceptable but with respectively lower activity of the protease and an impaired restart of migration.

Other proteases were tested: α-chymotrypsin (Sigma C 7762; buffers of steps 2 and 3: 0.2 M Tris-HCl, pH 8.8), papain (Sigma P 4762; 0.022 M KH2PO4, 0.048 M Na2HPO4, pH 7.2), Streptomyces griseus protease (Sigma P 0652; glycine, 0.051 M NaCl, NaOH, pH 11), Staphylococcus aureus V8 protease (Sigma P 8400; 0.2 M Tris-HCl, pH 7.5), and elastase (Sigma E 1250; 0.2 M Tris-HCl, pH 8.8). For papain, elastase, and Streptomyces griseus protease, the restart of migration was bad, and it was impossible to assess whether digestion had occurred; other buffers in steps 2 and 3 have yet to be tested. With Staphylococcus aureus protease, no digestion products were observed with enzyme concentrations up to 150 µg/100 mL; restart of migration was good. Only faint proteolytic patterns were observed even at high concentration of α-chymotrypsin (200 µg/100 mL).

Fig. 1c shows a gel treated by the standard method. On restarting electrophoresis in the opposite direction to the original separation, the proteolytic products of each polypeptide were observed above the original spot. Of the 450 spots visible in regular gels, about 250 were still visible after proteolysis. The absence of numerous spots, and the reduced intensity of most of them, was mainly due to protease activity since, otherwise, almost all spots retained approximately their original intensity in the control gels (Fig. 1b). About 75% of these 250 spots showed a pattern of proteolysis (even if fuzzy). Most of the remaining 25% were also digested, since their intensity decreased markedly, but they were too small for the digestion products to be seen. Nevertheless, few polypeptides were clearly not digested by ficin: their intensity was similar to that in the control gel, and there were no traces of digestion products above them. Some relatively large spots not degraded by ficin are shown in Fig. 1c.

In addition to the selection of protease, several parameters can be changed to improve the proteolytic patterns or their reproducibility. At higher protease concentration the number of digestion products is increased (Fig. 2), but at levels that were too high, a background appeared, visible e. g. for ficin at a concentration of 500 µg/100 mL. When the patterns of several spots are superimposed, the migration of step 5 can be performed in another direction, although a second front appears when the direction is not reversed (Fig. 3). The duration of the second migration can be optimized for specific spots. The pattern of some spots can never be correctly observed, especially in the case of high density of relatively small spots. In such a case it may be preferable to use other protein extracts from other organs or subcellular fractions with a resultant decrease of spots in the vicinity of spots of interest.
3.2 Comparison of proteolytic patterns

With the standard method (Section 2.3), the number of spots per proteolytic pattern was between 1 and 6. Two proteolytic patterns were considered as similar when they had the same number of spots, with similar relative positions and intensities, and when this was reproducible on at least 3 gels. With the silver staining method used here, only few spots had specific colors but this was useful in identifying profiles. Thirty-one groups of “Chinese Spring” leaf polypeptides were identified according to the similarity of their proteolytic patterns (Fig. 4) with seventy-six spots grouped. Similar experiments with etiolated seedlings allowed the confirmation of 17 groups (proteins present in both tissues) and the observation of 7 new groups (proteins either absent or barely visible in patterns of leaf proteins). By studying two organs, 100 spots were assigned to similarity groups. However, this does not represent the total information brought by the method, since the absence of similarity between two given spots is also valuable (and actually more easily proved).

All groups contained 2–5 polypeptides. As “Chinese Spring” is a homozygous line of a hexaploid species, homeoeallelic products were expected to give rise to groups of 1 to 3 spots in the absence of posttranslational modifications [12]. Thus, it is likely that at least the groups of more than 3 polypeptides contain products of posttranslational modifications. In general, this technique gives no information by itself on the causes of the similarities observed, but it can be used to verify hypotheses deduced from other experiments. For example, spots C1 and C2 were suspected to correspond to two products of the same cytoplasmic gene because of their similar behavior according to the cytoplasmic genome [13] (the third spot of this group was not considered in this study because it was not reproducible enough). The similarity of their digestion profiles observed by this technique reinforced this hypothesis. In this case, their structural similarity was finally proved by immunological techniques: they correspond to different forms of the β subunit of the chloroplastic ATP synthase.

4 Concluding remarks

Peptide mapping of proteins, digested by proteases directly in 2D gels can quickly provide valuable information on the relationships between polypeptides. It can be used, when additional spots are observed after protein purification, to test whether they are contaminants or modifications of the same protein. Another application is the comparison between products of in vitro translation and products of in vivo synthesis. It can also be particularly useful in phylogenetic studies for the comparison between 2D patterns of different species.

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Figure 4. Groups of related polypeptides in “Chinese Spring” leaf proteins. Polypeptides belonging to the same group according to their proteolytic pattern are marked with a star and linked by a solid bar. C1 and C2: two forms of the β subunits of the chloroplastic ATP synthase.
Characterization of human skin fibroblast extracellular proteins by two-dimensional polyacrylamide gel electrophoresis

Human skin fibroblasts secrete over 50 proteins into the culture medium. In this paper these are characterised using two-dimensional polyacrylamide gel electrophoresis and peptide mapping of proteins metabolically labelled in the presence and absence of tunicamycin. Thirty of these proteins have been shown to be N-glycosides, 4 are O-glycosides and 10 are not glycosylated. Of the major proteins, groups 1–4 have previously been shown to be fibroblast specific. Peptide mapping and tunicamycin treatment has identified that groups 1 and 2, and groups 1 and 3 arise by N-glycosylation of 2 and 4, respectively. The unglycosylated precursor forms of several other proteins have also been identified. This approach to the analysis of protein secretion provides an abundance of information on many proteins simultaneously and can be used to assess the changes in protein secretion associated with development, and to identify extracellular growth factors and other regulatory proteins.

1 Introduction

Human skin fibroblasts are commonly used for research into cellular function and to study the molecular basis of many diseases. However, little is known about fibroblast extracellular proteins or the distinction of fibroblasts from different tissues. Many proteins contain non-amino acid components attached to the polypeptide chain. The most common of these are the oligosaccharide units which occur in glycoproteins. These side chains can determine a wide range of biological properties e.g. intracellular location; structural and immunological characteristics; and proteolytic susceptibility [1, 2]. Glycosylation is often necessary for secretion into the extracellular environment and all the proteins in human serum are glycosylated during their biosynthesis with the exception of albumin and some of the lipoproteins [3]. Glycoproteins such as fibronectin, laminin and collagens are important constituents of the extracellular matrices of various cell types and are involved in cell adhesion, differentiation and growth [4].

Glycoproteins can be divided into two broad groups according to the mode of attachment of carbohydrate to protein [5]. In both groups the anemic carbon atom of the first carbohydrate residue is linked by a condensation reaction to an amino acid in the polypeptide chain. In O-glycosides, the attachment site is the hydroxyl group of a serine, threonine or hydroxylysine residue. In N-glycosides, more common in mammalian cells, the glycosidic bond is formed with the amido group of an asparagine residue.

N-Glycosides share a common carbohydrate precursor core structure:

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\begin{align*}
M & \quad 1 \\
M & \quad 3 \\
M & \quad 6 \\
\end{align*}
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Abbreviations: ASN, asparagine; DMD, Duchenne muscular dystrophy; DMEM, Dulbecco's modified eagle's medium; 2D-PAGE, two-dimensional polyacrylamide gel electrophoresis; FCS, fetal calf serum; GN, N-acetyl-D-glucosamine; M, D-mannose; SDS, sodium dodecyl sulphate; T+ cells, tunicamycin treated cells

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References
