USE OF DISK ELECTROPHORESIS FOR THE SEPARATION OF LARGE PEPTIDE FRAGMENTS OF PROTEINS

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Disk electrophoresis and isoelectric focusing in polyacrylamide gel can be used for the isolation of homogeneous proteins in amounts sufficient for their primary chemical characterization and, in particular, for the determination of their amino-acid composition [1] or N-terminal sequence [2]. The present paper describes a method of extracting from gels colored proteins and their large peptide fragments which enables several milligrams of homogeneous preparation, separated from the acrylamide, dyes, components of buffers, and detergents, to be isolated. The preparations so obtained can be used for the analysis of amino-acid composition, terminal groups, and other characteristics. The method is particularly convenient for monitoring the course of the separation of large peptide fragments in an analysis of the primary structure of a protein.

This method is based on the extraction with formic acid of the large peptide fragments after their separation by disk electrophoresis. The choice of formic acid was governed by the fact that, being a good solvent of proteins and peptides, it does not interfere with their integrity and, consequently, such proteins (peptide fragments) can be used for structural investigations. In addition, in formic acid it is easy to separate dyes from the protein. After evaporation of the formic acid and dissolution of the residue in a volatile alkaline buffer (since at an acid or neutral pH the incomplete dissolution of peptide fragments may be observed), the dye is separated on a column of Sephadex G-25.

By using the method described we have investigated a series of peptide fragments obtained in the treatment of carboxymethylated porcine pepsin with cyanogen bromide. It is known that the C-terminal fragment of pepsin, B1, possesses a tendency for pronounced aggregation [3]. On the disk electrophoresis of the mixture of peptides, a fraction was found which did not even pass into the 5% gel and collected at its boundary (Fig. 1A). This behavior is characteristic for proteins with a molecular weight greater than 500,000. This fraction was extracted from the gel and its amino-acid composition was determined, proving to be practically identical with that of the peptide B1. The partial disaggregation of peptide B1 can be achieved by treating it with a 1% solution of Tween 80 and by disk electrophoresis in gels containing 1% of this detergent.

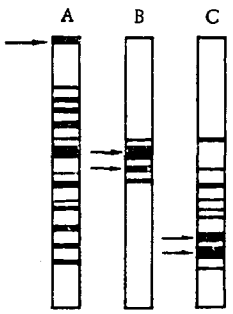

Fig. 1. Results of the disk electrophoresis of a mixture of cyanogen bromide peptides of porcine pepsin (A), of a preparation of the B2 fragment (B), and of a preparation of the B4 fragment (C): A, B) 15% polyacrylamide gel, C) 22.5% acrylamide gel (the arrows denote the fractions corresponding to B1, B2, and B4).

We also studied a fraction enriched in the peptide B4. In the experiment we used 3.5 mg of B4 (this corresponds to a load in one gel of about 300 μg when 12 gels are used simultaneously). In the unpurified B4 preparation nine peptide fractions were revealed, and these were investigated. Amino-acid analysis showed that B4 consists of two fractions because of the presence at the C-end of this peptide of homo-

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TABLE 1. Amino-Acid Compositions of the B1, B4, and B2 Fragments

Amino acid	B1		B4		B2	
	E	S	E	S	E	literature [8]
Aspartic acid	5	5	6	7	22	23
Threonine	2,5	2	3	3	15	15
Serine	4	2	7	7	24	24
Glutamic acid	3	2	3,5	4	15	16
Proline	2	2	3	3	7—8	7
Glycine	5	4	5	4	21	20
Alanine	3	3	1,5	2	8	8
Valine	5	5	2	2	10	9
Isoleucine	2	2	3,5	4	13	12
Leucine	3	3	3	3	15	15
Tyrosine	2	2	1,5	2	7,5	8
Phenylalanine	1,5	2	2	2	7	7
Lysine	1	1	—	—	—	—
Histidine	—	—	—	—	1	1
Arginine	2	2	—	—	—	—

Note. E is the amino-acid composition of the peptide isolated electrophoretically; S is the amino-acid composition calculated from the structure of the peptide.

serine, the carboxy group of which may be free (consequently, under the experimental conditions this fraction possesses a greater mobility) or may be in the form of a lactone (which leads to the appearance of a fraction with a lower mobility) (see Fig. 1B) [4].

Since the fragments B1 and B4 have low molecular weights, it appeared of interest to check the method on a large peptide. For this purpose we selected the fragment B2 occupying the N-terminal position in the molecule of pepsin and numbering about 200 amino-acid residues (i.e., as many residues as many peptides contain) (see Fig. 1C) [5]. The N-terminal amino-acid of this peptide, like that of pepsin, is isoleucine. As the results of an investigation of the fraction extracted after disk electrophoresis from the gel and corresponding in electrophoretic mobility with fragment B2 showed, the N-terminal amino acid of this peptide determined by the dansylation method is also isoleucine. The amino-acid composition of the fraction isolated does not differ from the composition of fragment B2 obtained previously by chromatographic methods. On the basis of these results it may be concluded that the fraction isolated from the gel was the cyanogen bromide fragment B2. On electrophoresis, peptide B2 gave two fractions, which may also be due to the presence of B2 of homoserine and its lactone at the C end.

Table 1 gives the amino-acid compositions of the fragments B1, B2, and B4 obtained by chromatographic and electrophoretic methods. On comparing the compositions of the peptides, it can be seen that the results of the present work agree well with those obtained previously. Some differences may be connected with the destruction of amino acids during acid hydrolysis (for example, tyrosine), with the possible sorption by the peptides of components of the electrophoretic buffer systems (for example, glycine), and also with the incomplete hydrolysis of some peptide bonds (for example, valylisoleucine).

The results that we have obtained show the reliability of the method described and the possibility of using it for investigating both proteins and peptide fragments of low molecular weight.

Thus, by disk electrophoresis with the subsequent extraction of the fractions from the gels with formic acid it is possible rapidly to isolate micro amounts of proteins or peptides for determining their amino-acid composition, for analyzing their N- and C-terminal sequences, etc. In our opinion, the method described may be a useful addition to the available methods for the separation and chemical characterization of proteins.

EXPERIMENTAL METHOD

The experiments were performed with acrylamide from Koch-Light, England; N,N'-methylenebisacrylamide, tris, N,N,N',N'-tetramethylethylenediamine, and glycine from Reanal, Hungary; sucrose and urea from BDH, England; Sephadex G-25 from Pharmacia, Sweden; Coomassie Blue GL from Serva, GFR; and sodium dodecyl sulfate, Tween 80, and Triton X100 from Ferak, Berlin. All the other reagents were analytically pure.

The fragments of carboxymethylated porcine pepsin were obtained by cleavage with cyanogen bromide at a ratio of cyanogen bromide to protein of 500 : 1 and were separated on Sephadex G-200 [6].

Disk electrophoresis was performed on a Canalco-Europe instrument (Holland) in glass tubes (6 × 70 mm) using a tris-glycine system of buffers with a separation pH of 9.5 [7]. For analyzing the peptides we used separating polyacrylamide gels with concentrations of acrylamide of 15 and 22.5% (ratio of acrylamide to methylenebisacrylamide 200 : 1), and a concentrating gel with an acrylamide concentration of 2.5% (ratio of acrylamide to methylenebisacrylamide 4 : 1). For the better dissolution of the peptides while avoiding their aggregation, the polyacrylamide gels were prepared on the basis of concentrated solutions of urea (final concentration of urea in the gel 6 M). To prepare the gels, instead of water we used a 10 M solution of urea to which tris-glycine buffer, a solution of acrylamide, and methylenebisacrylamide had been added,

and also ammonium persulfate and N,N,N',N'-tetramethylethylenediamine to the required concentration [7]. We used only freshly prepared urea solutions since cyanate – a product of the rearrangement of urea – can react with the amino groups of peptides [8]. The electrode buffers did not contain urea. Gels containing detergents – sodium dodecyl sulfate, Triton X100, and Tween 80 (the final concentration of detergents in the gel being 1%) – were prepared similarly.

The protein samples (200-300 μg per gel) were deposited in a 6 M solution of urea (or in a 1% solution of the appropriate detergent) containing 10% of sucrose. Bromophenol Blue was used as the marker of the electrophoretic front. Electrophoresis was performed at a current strength of 4 mA/gel for 1-1.5 h (in the first 10 minutes, the current strength was 1 mA/gel). The gel was fixed in 30% trichloracetic acid for 1 h and was stained with a 0.5% solution of Coomassie Blue GL in 30% trichloroacetic acid for an hour. The excess of dye was eliminated in 7.5% acetic acid.

After the elimination of the excess of dye, the stained bands corresponding to the fractions of the peptides were cut out and comminuted. The peptides were extracted from the gels with 70-75% formic acid at −15°C with stirring for 14-15 h. For complete extraction, the volume of the formic acid must be at least 10 times the volume of the gel segments. Extraction was performed three times. To eliminate the polyacrylamide, the extracts were filtered through a glass filter of medium porosity, combined, and rapidly evaporated in a rotary evaporator at 25-30°C. Then the residue was dissolved in 1.0-1.5 ml of triethylammonium carbonate buffer (pH 8.5) and the dye was separated in a 10-ml column containing Sephadex G-25 equiliberated with the same buffer. The issue of peptides from the column was recorded by means of a Uvicord II flow-through ultraviolet spectrophotometer (LKB, Sweden). In the process of chromatography, the peptides were separated from the dye, residues of detergents, the components of the electrophoretic buffer, etc., and were obtained in amounts ranging from a few hundred micrograms to several milligrams with a yield of 90-95%.

Acid hydrolysis was performed with 5.7 N hydrochloric acid for 24 h under vacuum, and amino acid analysis was carried out on a BC-200 analyzer (Bio-Cal).

SUMMARY

The disk electrophoresis of peptide fragments from porcine pepsin obtained by cleaving the pepsin with cyanogen bromide has been performed. A method has been developed for extracting the colored peptide fractions after electrophoresis in polyacrylamide gel. An amino-acid analysis has been performed of peptide fragments B1, B2, and B4 isolated by disk electrophoresis. The results obtained have been compared with the amino-acid composition calculated from the structures of these peptides. The possibility has been shown of using the method described during structural investigations of both proteins and their low-molecular-weight fragments.

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