Peptide Mapping by Limited Proteolysis in Sodium Dodecyl Sulfate and Analysis by Gel Electrophoresis*

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A rapid and convenient method for peptide mapping of proteins has been developed. The technique, which is especially suitable for analysis of proteins that have been isolated from gels containing sodium dodecyl sulfate, involves partial enzymatic proteolysis in the presence of sodium dodecyl sulfate and analysis of the cleavage products by polyacrylamide gel electrophoresis. The pattern of peptide fragments produced is characteristic of the protein substrate and the proteolytic enzyme and is highly reproducible. Several common proteases have been used including chymotrypsin, Staphylococcus aureus V8 protease, and papain.

Electrophoresis in gels containing SDS is a powerful tool for the separation of polypeptide chains in complex biological samples (1-3). Often, however, the unambiguous identification of relationships among specific proteins cannot be made on the basis of electrophoretic mobility alone. This may be true, for example, if closely related proteins differ in mobility due to slight chemical modifications, if larger and smaller peptides are related by a precursor-product relationship, or if related proteins differ in mobility as the result of artifactual proteolysis during preparation. In such cases it becomes desirable to subject individual protein bands to further biochemical analysis. Indeed, methods have been described for the isolation of proteins from individual gel bands, thereby permitting their use in renaturation and enzymatic activity studies (4), in amino acid analyses (5), in two-dimensional peptide mapping (6), and (as antigens) in immunological studies (7). We report here on a new, readily applicable procedure for peptide analysis of proteins. The method, which is especially suitable for analysis of proteins which have been isolated from SDS gels, involves the partial digestion of proteins by any of several proteases in a buffer containing SDS. Relatively stable partial digests are produced, composed of many peptides whose molecular weights are sufficiently large that their separation on 15% acrylamide-SDS gels is possible. The pattern of the bands of peptides so generated is characteristic of the protein substrate and the proteolytic enzyme and is highly reproducible.

Moreover, the technique can be completed in a matter of hours and requires as little as 5 to 10 μg of protein.

Materials and Methods

Enzymes and Substrates—Bovine serum albumin (A-4503) and papain (A-4762) were obtained from Sigma. Escherichia coli alkaline phosphatase (BAPC-5129) and chymotrypsin (CDI-1450) were purchased from Worthington, Staphylococcus aureus V8 protease (36-900-1) was obtained from Miles Laboratories. Tubulin was prepared from microtubule protein by chromatography on phosphocellulose as described by Weingarten et al. (8). The precursor protein P23 of the major head protein of bacteriophage 14 was isolated from cells infected with phage containing a mutation (N54) in gene 31 (9) and the cleavage product, P23*, was isolated from filtered phage particle (10).

Gel Electrophoresis—Electrophoresis in gels containing SDS was performed in a slab gel apparatus (10) utilizing the discontinuous system described by Laemmli (3). For analysis of peptides generated by proteolysis, 15% acrylamide gels (30:0.5 by weight acrylamide:bisacrylamide) were routinely used. Gels were stained in a solution containing final concentrations of 0.1% Coomassie blue, 30% methanol, and 10% acetic acid, and destained by diffusion in a solution of 5% methanol and 10% acetic acid.

Electrophoretic Elution of Stained Proteins from Gels—Bands of interest were visualized in preparative gels by staining. To avoid possible acid hydrolysis, gels were stained for no more than 30 min and destained for less than 1 h. The bands were then cut from the gel and the protein was eluted by electrophoresis overnight into a dialysis bag. The contents of the dialysis bag were cooled to 4° and then precipitated by the addition of trichloroacetic acid to a final concentration of 20%. The precipitate was pelleted by centrifugation, washed twice with ethyl ether, and resuspended in the final sample buffer.

Digestion Procedure for Purified or Eluted Proteins—Purified proteins or proteins eluted electrophoretically from gels were dissolved at approximately 0.3 mg/ml in sample buffer which contained 0.125 M Tris/HCl at pH 6.8, 0.5% SDS, 10% glycerol, and 0.0001% bromphenol blue. The samples were then heated to 100° for 2 min. Proteolytic digestions were carried out at 37° for 30 min by addition of given amounts of any of several proteases as indicated in the appropriate figure legends. Following addition of 2-mercaptoethanol and SDS to final concentrations of 10% and 2%, respectively, proteolysis was stopped by boiling the samples for 2 min. About 20 to 30 μl (10 to 15 μg) of each sample were loaded into a sample well of the 15% acrylamide-SDS gel and then overlaying each slice with protease. Digestion proceeded directly in the stacking gel during the subsequent electrophoresis. The best results were achieved using 1 mM EDTA in the gel solutions on 1.5-mm-thick gels which were cast with longer than usual stacking gels (up to 5 cm) and wider than...
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Reproducibility and Stability of Proteolysis Products—To test the reproducibility of the pattern of peptide bands produced from a given protein by proteolysis in our SDS-containing buffer, *Escherichia coli* alkaline phosphatase was digested for various incubation times and at various enzyme concentrations with the *Staphylococcus aureus* V8 protease. (This protease cleaves at the COOH-terminal side of aspartic and glutamic acid residues (11) and has proven to be eminently suitable for generating digests which contain many appropriately sized peptide fragments.) Fig. 1A shows the digestion at fixed enzyme concentration for incubation times between 0 and 150 min. Alkaline phosphatase, whose apparent molecular weight on SDS gels is about 50,000, is labeled with the letters AP. The *Staphylococcus aureus* protease generates a doublet, labeled E, which runs slightly faster than the alkaline phosphatase band. It can be seen that with increasing time of digestion there is a progressive disappearance (complete after 30 min) of the alkaline phosphatase band. Depending on the length of incubation, about 10 to 15 well resolved cleavage fragments can be observed. The apparent molecular weights of the fragments range from 6,000 to 30,000. Although some bands existing at early times disappear upon further digestion and others are generated only at later times, the stability of many of the cleavage fragments is remarkable. Of the 10 fragments which can be seen following a 15-min incubation period, all but two can still be detected after 60 min, with two to three new fragments appearing during this time period.

The digestion of alkaline phosphatase using increasing *Staphylococcus aureus* protease concentrations, but at a fixed incubation time of 150 min, is shown in Fig. 1B. The increase in the intensity of the protease doublet at higher enzyme concentrations is evident. It can also be seen that the same peptide fragments which were present in the time course experiment (Fig. 1A) are again observable in this experiment. As expected, bands corresponding to larger peptides are lost at increasing protease levels, with most of the higher molecular weight fragments disappearing at enzyme concentrations above 20 μg/ml.

In a separate experiment (not shown), the peptide pattern produced by digestion of albumin with a constant concentration of chymotrypsin was observed to be unaffected by 16-fold variation in albumin concentration between 0.16 mg/ml and 2.56 mg/ml. These results collectively demonstrate that reproducible cleavage fragments of suitable size are generated by proteolytic digestion of a substrate protein in our standard SDS-containing buffer.

**Fig. 1.** Kinetic study of digestion of alkaline phosphatase. A, alkaline phosphatase (labeled AP) at a concentration of 0.33 mg/ml in sample buffer was digested at 37° with a final concentration of 25 μg/ml of *Staphylococcus aureus* protease (labeled E). The length of incubation was varied between 0 and 150 min as indicated in the figure. Twenty microliters were loaded per sample well. B, alkaline phosphatase at a concentration of 0.33 mg/ml in sample buffer was digested at 37° for 150 min with increasing concentration of *S. aureus* protease, as indicated in the figure in micrograms/ml. Twenty microliters were loaded per sample well. Four micrograms of *S. aureus* protease, incubated at 200 μg/ml in the absence of alkaline phosphatase, were run in the final slot on the right.
FIG. 2 (top). Peptide maps of albumin, tubulin, and alkaline phosphatase. Albumin, tubulin, and alkaline phosphatase, each at a concentration of 0.67 mg/ml in sample buffer, were incubated at 37°C for 30 min with concentrations of protease as indicated. Thirty microliters of sample were loaded per sample well of a 20% gel. A to C, digestions of albumin, tubulin, and alkaline phosphatase with final concentrations of 33 μg/ml, 3.3 μg/ml, and 33 μg/ml, respectively, of papain. D to F, digestions of albumin, tubulin, and alkaline phosphatase with 133 μg/ml, 67 μg/ml, and 67 μg/ml of Staphylococcus aureus protease. G to I, digestions of albumin, tubulin, and alkaline phosphatase with 133 μg/ml, 67 μg/ml, and 67 μg/ml of chymotrypsin. J to L, undigested albumin, tubulin, and alkaline phosphatase. A total of 2.5 μg was loaded per sample well. M to O, highest total amounts of protease used in the digestions—1 μg of
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line Phosphatase with Different Enzymes—To assess the usefulness of this technique in clearly distinguishing various proteins, the digestion patterns of three different substrate proteins produced with three different proteases were examined. The conditions of the digestions were chosen in each case so that partial digests, which were insensitive to at least small changes in enzyme concentration or incubation time, were produced.

The peptide patterns of albumin, tubulin, and alkaline phosphatase, which resulted from digestion with papain, are shown in Fig. 2, A, B, and C, respectively. Although roughly 15 bands are observable for each substrate, the three patterns are strikingly distinct, with essentially no bands appearing in common between any pair. This specificity is further underscored in Fig. 2, D, E, and F, where these same three substrates have been incubated with the Staphylococcus aureus protease and in Fig. 2, G, H, and I, in which the digestions have been effected with chymotrypsin. Not only are the banding patterns produced in these examples again unique to the protein substrate, but furthermore, by comparison of the patterns generated from a single substrate by the action of the three different proteases (e.g., the albumin patterns in Fig. 2, A, D, and G), it can be seen that the patterns produced from a particular substrate are unique for each of the proteases utilized as well.

Comparison of Digestion Products of α and β Tubulin Eluted from SDS Gel—To illustrate the application of the present technique of peptide mapping to proteins which have been obtained by electrophoretic elution from SDS gels, we chose to analyze the two polypeptide chains, α and β, of tubulin. These two chains, which have similar amino acid compositions and related NH₂-terminal sequences (12) and which exist together as a stable dimer under native conditions, may be separated under denaturing conditions on SDS gels into two distinct bands with respective apparent molecular weights of 53,000 and 55,000. In the present experiment, the α- and β-bands were cut from a preparative gel and eluted electrophoretically. Fig. 3, A and R show the re-electrophoresis of the separated α- and β-subunits, while Fig. 3C illustrates the equimolar presence of both bands in a gel slot loaded with a sample of the tubulin dimer. The banding patterns of the peptides generated by chymotryptic digestion of the isolated α- and β-tubulins are given in Fig. 3, D and E. It is notable that the two patterns are completely distinct, with each pattern containing many peptides of mobility not present in the other. Fig. 3F shows the digestion products of the pure tubulin dimer produced with chymotrypsin. As expected, this pattern is seen to be simply a composite of the peptide fragments observed in the digestions of the separated α- and β-tubulins (Fig. 3, D and E). An identical experiment is shown in Fig. 3, G, H, and I, with the exception that digestions were performed with the Staphylococcus aureus protease.

Proteolysis during Re-electrophoresis—In order to avoid an elution step for those proteins whose study requires their isolation from SDS gels, we have developed a method in which a protein band of interest may be cut from a preparative gel and subsequently applied directly to a high per cent acrylamide gel in the presence of a proteolytic enzyme. This procedure generates banding patterns which are identical to those obtained from the same protein (prepared by elution from preparative gels or purification with classical biochemical techniques) after digestion in solution prior to loading onto the high per cent gel.

An example of the method is shown in Fig. 4. The protein P23 (molecular weight 55,000) is the precursor protein of P23* (molecular weight 45,000), the major head protein of bacteriophage T4 (3). The cleavage fragment with molecular weight about 10,000 is known to be removed during head maturation and is derived from the NH₂-terminal end of the protein (13). Comparison of the digestion pattern of the precursor protein P23 and its product protein P23* would be expected to show (a) a large number of common peptides, (b) the presence in the pattern of P23 of some peptides not found in the P23* pattern, and (c) possibly the presence of a peptide unique to the P23* pattern due to the formation of the new NH₂-terminal end. In the actual experiment (Fig. 4), the proteins P23 and P23* were re-electrophoresed with three different concentrations of Staphylococcus aureus protease. As expected, there were several bands (11 in Fig. 4, C and D) which are common to both proteins P23 and P23* and a few bands (4 in Fig. 4C) unique to the larger precursor, P23. One band is enriched in the digest of P23* and may, therefore, be the new NH₂ terminus.

DISCUSSION

These experiments demonstrate that proteolytic digestion of protein substrates in the presence of SDS results in the generation of many relatively large peptide fragments, which may subsequently be separated on high concentration acrylamide gels. In conventional peptide mapping, resolution is achieved by use of a two-dimensional system (electrophoresis and chromatography). The present method achieves comparable resolving power in one dimension by use of several proteases of different specificities, and if necessary, this resolution may be extended by further proteolysis of individual peptide frag-
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ments with additional enzymes. Indeed, the sensitivity of the method in identifying particular proteins has been clearly documented. Different protein substrates produce strikingly different patterns of digestion with a given protease, and similarly, unique patterns are produced from a single substrate by action of proteases of different specificities. In addition, although observable differences in the peptide patterns of α- and β-tubulin were not unexpected, since the cyogen-sort fragments of the two proteins are also distinct (19), the complete dissimilarity of the patterns generated does serve to illustrate the power of the technique in discriminating between two proteins which have similar amino acid compositions and which probably share a common evolutionary origin.

Reproducibility of the pattern of peptides is ensured by the marked insensitivity of the digests to large variations in protease and substrate concentrations and in the length of incubation with the protease. In addition, the digests are unaffected by the manner of preparation and are reproducible as demonstrated in Fig. 3. The α- and β-tubulin bands were purified by SDS-gel electrophoresis, fixed and stained with Coomassie blue in acetic acid and methanol, electrophoretically eluted from the gel, precipitated with trichloroacetic acid, and finally resuspended in SDS-containing sample buffer. Unfractionated tubulin was merely diluted into the sample buffer. Yet, the peptide fragments of the unfractionated tubulin are simply the sum of the fragments obtained with the α and β peptides. No extraneous bands were produced. Numerous analyses of α- and β-tubulin, serum albumin, and alkaline phosphatase have yielded reproducible peptide fragments. In addition to the protein digests reported here, the method has been used to show the similarity of actin from muscle and actin from cultured Chinese hamster ovary cells, to identify histone Fl from phsyarum, for identification of two adenovirus-specific DNA-binding proteins, a viral structural protein, and the adenovirus tumor antigen, to illustrate the power of the technique in discriminating between two proteins which have similar amino acid compositions and which probably share a common evolutionary origin.

The technique is quite versatile and simple to use. Several patterns can be obtained simultaneously in a matter of hours and can be displayed in the same slab gel, thereby allowing easy, unambiguous comparison. No special equipment beyond the standard slab gel electrophoresis apparatus is required. Furthermore, the proteases which have been found to produce suitable digests are all relatively inexpensive and commercially available.

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