BLOCKING OF TRYPTIC CLEAVAGE OF ARGINYL BONDS BY THE CHEMICAL MODIFICATION OF THE GUANIDO GROUP WITH BENZIL

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When benzil is added to an alkaline solution of arginine, the Sakaguchi reaction is abolished, and a new compound can be isolated in crystalline form from the reaction mixture. The elemental analysis of this product corresponds to an equimolar addition of benzil to arginine with loss of a mole of water.[1] Benzil and lysine do not form a stable product under the same conditions. In the present study proteins were reacted with benzil, and the products were digested with trypsin. The results indicate that the arginyl bonds of benzil-treated proteins become resistant to tryptic hydrolysis while the cleavage of lysyl bonds takes place in the usual manner.

The general procedure for reacting benzil with protein was to add a ten-fold excess of benzil to a solution of protein in 70-80% ethanol which was 0.2 molar in a strong base. The reaction mixture was allowed to stand 16-18 hours at room temperature (23-28°) under nitrogen and then neutralized. The solvent was evaporated under reduced pressure, and the salt and excess benzil removed by dialysis or extraction. The reagents and procedure were modified according to the properties of the material under investigation; for example, dialysis was avoided in the studies on insulin and salmine described below because of their low molecular weights. Tetraethylammonium hydroxide and

[1] This compound and the products of the addition of benzil to a variety of derivatives of arginine were prepared by one of us (H.A.I.) in the laboratories of Dr. S. Akabori at the Institute of Protein Research of Osaka University. These investigations will be reported elsewhere.

propionic acid were used for strong base and neutralizing acid, respectively, and their salt was extracted with isopropanol or acetone. The residue after extraction was dissolved or suspended in water and lyophilized.

Salmine[2] and performic acid oxidized insulin were chosen as model substrates. Oxidized insulin was prepared from crystalline beef insulin[3] by the method of Craig and Henry (1961). Insulin contains a residue each of arginine and lysine in its B chain while salmine is 85% arginine by weight and contains no lysine. Tryptic digestion was carried out at pH 8 and 38° in the reaction cell described by Merigan et al. (1962) and monitored on the "pH stat". TCA trypsin[4] was used in a weight ratio of one part by weight to forty of substrate. Peptide analysis was performed by a fingerprinting method (Katz et al., 1959), and by one-dimensional high voltage paper electrophoresis in pyridine acetate buffer of pH 3.6 at 5000 volts for 4 hours at 20° C. Following fingerprinting or electrophoresis the dried papers were stained lightly with ninhydrin and the stained areas eluted. After evaporation to dryness the residues were subjected to anaerobic hydrolysis in 6 N HCl. The amino acid composition was determined by an electrophoretic technique (Dreyer and Bynum, 1960) and by the color reactions for arginine, histidine, and tyrosine.

When tryptic digests of oxidized insulin were fingerprinted, three discrete spots were detected. They were identified by their amino acid compositions as the three fragments of the B chain that result from cleavage at its arginyl-glycyl and lysyl-alanyl bonds. The relatively large unbroken A chain was barely discernible as a diffuse spot which moved the slowest in chromatography and streaked on electrophoresis.

[2] Available as protamine sulfate from Sigma Chemical Company. St. Louis, Missouri.

[3] A gift of Dr. O. K. Behrens of the Eli Lilly Company. Indianapolis, Indiana. Lot #693502.

[4] Biochemical Corp., Freehold, New Jersey. Worthington Lot #591.

Only free alanine was identifiable by electrophoresis in the tryptic digest of benzil-treated oxidized insulin. Two other spots were detectable after chromatographic separation of this digest, one which behaved like the A chain and another which was fluorescent. The two peptides which would result from cleavage of the arginyl-glycyl bond were not seen.

The product of the treatment of salmine with benzil is a fluorescent, rather insoluble material with a lower cationic mobility than untreated salmine and a negative Sakaguchi reaction. Base uptake on treatment of this product with trypsin was negligible (Fig. 1). No new components were detectable by paper electrophoresis, and the mobility of benzil-treated salmine before and after treatment was the same. Acid hydrolysis and amino acid analysis revealed the disappearance of free arginine and the appearance of a ninhydrin-positive spot having the mo-

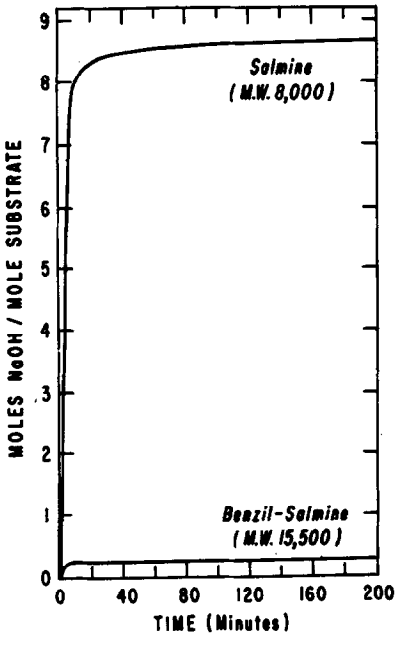

Figure 1. Course of the tryptic digestion of salmine and benzil-treated salmine.

bility of the crystalline addition product of benzil and arginine. No other change in amino acid content was observed.

The results indicate that the use of a strongly alkaline medium for the reaction of benzil with protein does not result in detectable nonspecific hydrolysis of peptide bonds. Arginine residues are quantitatively modified by this reaction, and other amino acid residues are unaffected. Since the modification results in a specific block of tryptic hydrolysis of arginyl bonds, its use will facilitate the determination of "arginine overlaps" in studies of the primary structure of peptides and proteins. Subject to reversibility of the denaturation which accompanies the procedure, this method of protein modification may also be applicable to enzymatic and immunochemical studies.

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