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Ovalbumin Is an Elastase Substrate*

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Ovalbumin is partially homologous in sequence with the proteinase inhibitors α_1-proteinase inhibitor and anti-thrombin III. The region of sequence in ovalbumin which corresponds to the reactive sites of these proteinase inhibitors is susceptible to attack by subtilisin, elastase, thermolysin, bromelain, and *Bacillus cereus* protease. The esterase activity of elastase is not inhibited by ovalbumin, but ovalbumin is efficiently cleaved by elastase. In contrast with these proteases, trypsin does not cleave ovalbumin.

It has been known for many years that limited proteolysis of ovalbumin by subtilisin results in a nicked form of ovalbumin, called plakalbumin, which has a characteristic crystal form (1). Plakalbumin differs from ovalbumin in that an internal heptapeptide has been released in several fragments by the proteolysis (2). The biological function of ovalbumin is not known, but a search for sequence homologies by Hunt and Dayhoff (3) found a homology among α_1-proteinase inhibitor, anti-thrombin III, and ovalbumin. The reactive sites of these inhibitors lie in the same sequence where subtilisin cleaves ovalbumin to form plakalbumin. Doolittle (4) has also found that angiotensinogen is homologous with this family of proteins.

As part of a crystallographic study of plakalbumin, which was initiated by Miller *et al.* (5), I have examined the sensitivity of ovalbumin to proteolysis by different proteases. The results of these digestion studies are described here, as well as the characterization of the steady-state kinetics of the elastase-catalyzed hydrolysis of ovalbumin.

MATERIALS AND METHODS

Ovalbumin, trypsin, α-chymotrypsin and *Bacillus cereus* (strain BRL-70) protease (microprotease) (6) were from Worthington; *N*-carbobenzoxy-L-alanine *p*-nitrophenyl ester, subtilisin BPN′, thermolysin, elastase, *Streptomyces griseus* protease, and bromelain were from Sigma; meprin, cathepsin L, and cathepsin B were gifts from Dr. Judy Bond of this department. CM-Sepharose was from Pharmacia.

Protease Digestion of Ovalbumin—Ovalbumin was digested in either 50 mM sodium cacodylate buffer (pH 5.4), 10 mM $CaCl_2$, or in 50 mM Tris (pH 8.0), 10 mM $CaCl_2$. Solutions of 200 μl of ovalbumin (10 mg/ml) and 20 μl of protease (1 mg/ml) in the selected buffer were mixed and kept at room temperature for 24 h. Samples were removed at 5, 10, and 24 h. Digestion was stopped by mixing the sample with an equal volume of a denaturing solution consisting of 2% sodium dodecyl sulfate, 20% glycerol, 125 mM Tris (pH 6.8), 0.02% bromphenol blue. These samples were then boiled and either frozen or run immediately on 15% polyacrylamide gels with 2-mercaptoethanol in the samples according to the procedure of Laemmli (7). Plakalbumin was prepared by digestion of a solution of ovalbumin (50 mg/ml) in 50 mM sodium cacodylate (pH 6.4) with subtilisin BPN′ (40 μg/ml) for 5 h at room temperature. The reaction was stopped by passing this solution over a column (25 × 6 cm) of CM-Sepharose in 50 mM sodium cacodylate (pH 5.4) to remove subtilisin.

Elastase Assays—The hydrolysis of ovalbumin by elastase was followed in a pH-stat by measurement of the release of protons. Concentrations of ovalbumin in the range of 0.18 to 0.73 mM were adjusted to pH 8.0 with NaOH in unbuffered solution. A solution of 5 μg of elastase in water was added to give a final volume of 0.8 ml, and the initial rate of hydrolysis was determined from the volume of 0.2 mM NaOH solution added to maintain the pH. Elastase activity was also measured by following the hydrolysis of *N*-carbobenzoxy-alanine-*p*-nitrophenyl ester at $\lambda \approx 400$ nm in 50 mM Tris (pH 8.0), 10 mM $CaCl_2$. The concentrations of substrate varied from 16 to 67 μM, and that of elastase was 0.08 μM as determined from A_{280}.

Amino-terminal Analysis—The site of elastase cleavage was determined by amino-terminal analyses of control and elastase-proteolyzed samples of ovalbumin. A sample of ovalbumin digested as described above was lyophilized at the end of the digestion period. This sample was redissolved and analyzed by the Edman degradation procedure on a Beckman sequenator through three residues.

Protein Concentration—Protein concentration was determined from A_{280} measurements using published values for extinction coefficients: $\epsilon^{1\%} = 7.5$ for ovalbumin; $\epsilon^{1\%} = 17.1$ for elastase.

RESULTS

Three broad classes of proteases could be defined by their effect on ovalbumin as follows: 1) those which do not digest ovalbumin (trypsin, Clostridial protease, thrombin, plasmin, meprin, cathepsin L, and cathepsin B); 2) those which cause extensive degradation of ovalbumin (*Streptomyces griseus* protease, proteinase K, and subtilisin BPN′ after 24 h of digestion); and 3) those which were relatively specific in their digestion of ovalbumin (elastase, thermolysin, bromelain, *B. cereus* protease, and subtilisin BPN′ after 5 h of digestion). Those in the third class gave as the principal digestion product a large fragment closely similar, if not identical, to plakalbumin, which is produced by subtilisin digestion. Ovalbumin digestion products of proteases of classes 2 and 3 as well as ovalbumin treated with trypsin are shown in Fig. 1.

The relatively high specificity with which elastase, thermolysin, bromelain, and *B. cereus* protease cleave ovalbumin at or near the same site as subtilisin implies that this part of the ovalbumin sequence is exposed and susceptible to these proteases. This region of the ovalbumin sequence corresponds to the location of the reactive sites in α_1-proteinase inhibitor and anti-thrombin III (Fig. 2).

Because of the high specificity with which elastase cleaves ovalbumin and because the amino acid sequence around the bond cleaved suggests that ovalbumin may be a specific substrate or inhibitor of this protease, the steady-state kinetic parameters of the elastase-catalyzed proteolysis of ovalbumin were measured and found to be $K_M = 1.1$ mM and $k_{cat} = 10$ s^{-1}. These values are approximate, since the elastase concentration is based on A_{280} measurement, and the precise relationship between proton release and bond cleavage in ovalbumin is not known. Ovalbumin was tested as an inhibitor of the elastase-catalyzed hydrolysis of the synthetic substrate, *N*-carbobenzoxy-L-alanine *p*-nitrophenyl ester. There was no detectable inhibition of this reaction. However, it should be noted that k_{cat}/K_M for elastase cleavage of ovalbumin is an

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