An Inhibitor of Trypsin-Like Activity in Rat Liver* (34108)

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Numerous trypsin inhibitors of plant and animal origin have been described (1). There has been some controversy, however, regarding the presence of such an inhibitor in rat liver tissue. Finkenstaedt (2) reported that a 10,000*g* supernatant solution, prepared from a liver homogenate, inhibited the hydrolysis of benzoyl-L-argininamide hydrochloride monohydrate (BAA) and glycyl-L-tyrosinamide acetate (GTAA) by a liver enzyme preparation which sedimented at 10,000*g*. Similarly Blackwood and Mandl (3) demonstrated complete inhibition of tryptic hydrolysis of benzoyl-DL-arginine-β-naphthylamide (BANA) by a crude liver homogenate. In contrast, Bouma and Gruber (4) could show no inhibition of catheptic activity present in the lysosomal cellular fraction by the supernatant fraction when BAA was used as substrate. The present report confirms the presence of a protease inhibitor in liver and describes its subcellular localization and some of its properties.

Materials and Methods. Rats weighing 200–400 g were killed by decapitation. The liver of each rat was immediately perfused with cold 0.85% NaCl until blanched. All subsequent procedures were performed at 5°. The excised liver was freed of connective tissue then homogenized in a Potter-Elvehjem homogenizer with 0.25 *M* sucrose, 2 ml/g of wet tissue, until two complete strokes were made. The homogenate was centrifuged for 10 min at 5000*g*. The pellet, containing nuclei and debris, was discarded and the supernatant fluid S_I was centrifuged at 10,000*g* for 10 min. The pellet P_{II}, which contained both mitochondria and lysosomes, was resuspended in the original volume of 0.25 *M* sucrose, and the supernatant solution, S_{II}, was centrifuged at 100,000*g* for 1 hr after removal of an aliquot for analysis. The resulting pellet, P_{III}, which contained the microsomal fraction, was resuspended in 0.25 *M* sucrose and the supernatant solution, S_{III}, after removal of an aliquot for analysis, was adjusted to pH 4.0 by the slow addition of 0.25 *M* acetic acid to the solution, which was constantly stirred. The pH 4.0 mixture was centrifuged at 10,000*g* for 10 min. The pellet, P_{IV}, which contained the inhibitor activity, was dissolved by suspending it in a minimum volume of 0.05 *M* tris (hydroxymethyl) methylglycine (Tricine) buffer, pH 7.5, and readjusting the pH 7.5 with 0.2 *M* NaOH. The pH of the supernatant solution S_{IV} was also adjusted to 7.5 by the addition of 0.2 *M* NaOH.

Two assay methods for tryptic activity were used. The Kunitz (1) procedure, which uses casein as substrate, was followed without modification. In the Blackwood and Mandl method for assay of tryptic-like activity, using BANA as substrate, the final volume of the incubation mixture was adjusted to 3 ml instead of 2 ml in all but the initial experiments (Fig. 1) in order to accomodate the addition of trypsin inhibitor. It was found necessary to protect reagents and the reaction mixture from light. Tris-maleate buffer (5), 0.05 *M*, pH 5.2–8.6, was used in the study of the influence of pH on inhibitor activity. One unit of inhibitor activity is defined as a decrease in the hydrolysis of BANA equivalent to 0.200 optical density units in the modified assay method of Blackwood and Mandl.

Results and Discussion. Assay of the fractions obtained by differential centrifugation of rat liver homogenate showed the presence

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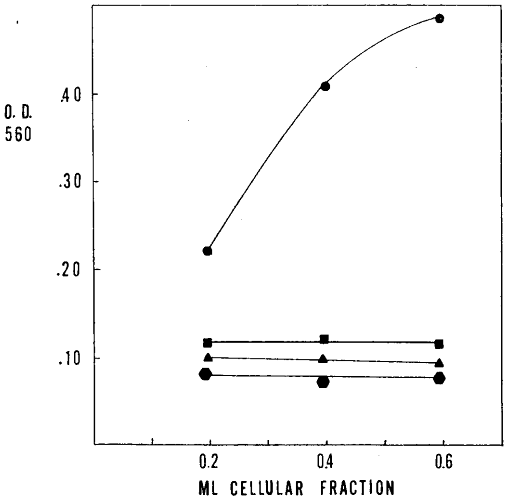

FIG. 1. Proteolytic activity of subcellular fractions. Each digestion mixture contained the subcellular fraction, 1 ml of BANA, and buffer, pH 7.5, to bring the total volume to 2 ml. Lysosomal-mitochondrial fraction P_{II} (●); supernatant fraction S_{II} (●); equal parts of fraction P_{II} and S_{II} (▲); and crude homogenate fraction S_I (■).

of proteolytic activity in the lysosomal fraction, P_{II}, and protease inhibitor in the supernatant solution of this fraction, S_{II} (Fig. 1). The supernatant solution from which these fractions were derived, S_I, showed no proteolytic activity. Inhibitor was shown to be present by its action on both lysosomal enzymes (Fig. 1) and on commercial trypsin (Fig. 2).

The inhibitory activity present in the supernatant S_{II} was further fractionated by centrifugation at 100,000*g*. Protease inhibitor was present in both the pellet (P_{III}, the microsomal fraction) and in the supernatant, S_{III}. Because S_{III} was readily soluble in aqueous solutions, whereas the inhibitor in P_{III} was solubilized only by repeated extraction with a 1% solution of nonionic detergent in 0.05 *M* Tricine buffer, pH 7.5, and because P_{III} and S_{III} represent different subcellular fractions, additional studies were performed with S_{III} only.

Purification of the inhibitor in fraction S_{III} by acid precipitation at pH 4.0 resulted in a sixfold increase in specific activity from 0.04 units per milligram protein to 0.25 units per milligram protein. The ability of this inhibitor preparation to inhibit tryptic hydrolysis of casein is shown in Fig. 3. The inhibitor was also found to be stable to heat (100° for 10 min) and was recovered within the dialysis bag after dialysis overnight at 5° in 3–4 liters of 0.01 *M* Tris-HCl buffer, pH 8.8, or 3–4 liters of distilled water.

The effect of pH on the inhibition by fraction P_{IV} of both lysosomal and tryptic hydrolysis of BANA is shown in Fig. 4. Inhibition of the lysosomal proteases increased progressively from less than 5% at pH 5.2 to nearly 70% at pH 8.4, whereas inhibition of trypsin by the same amount of fraction P_{IV} varied from less than 5% to 25% in the pH range tested.

It has been shown, then, that inhibitors of tryptic-like activity are present in the 100,000*g* fraction and in buffered detergent extracts of the microsomal fraction of rat liver tissue. The soluble 100,000*g* supernatant inhibitor is most active at physiologic and alkaline pH and may exhibit a regulatory control of protein turnover. After death,

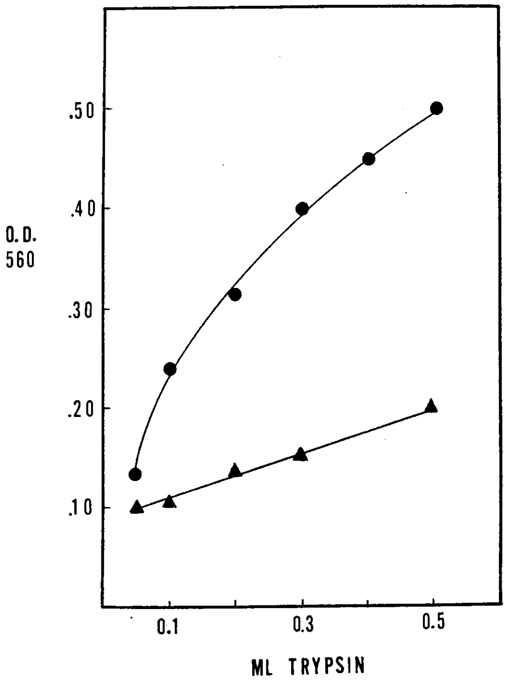

FIG. 2. Tryptic hydrolysis of BANA in the presence and absence of inhibitor, fraction S_{II}. Tryptic activity, 1 mg. trypsin/ml (●); tryptic activity, 1 mg. trypsin/ml in presence of 0.5 ml of fraction S_{II}(▲).

when the cellular environment becomes more acidic, the inhibitor is less active and lysosomal protease activity is accelerated. The fact that a substantial degree of inhibitor activity appears to be microsome-bound while the remainder is readily soluble suggests two possibilities. First, the microsomal inhibitor may be a progenitor of the soluble substance, bound to its site of origin prior to release into the cytoplasm. Second, the inhibitor may be present functionally at the microsomal membranes, serving to protect these essential cellular structures from autodigestion. Current investigations, involving isolation and characterization of the inhibitors present in the two fractions, will presumably resolve these possibilities.

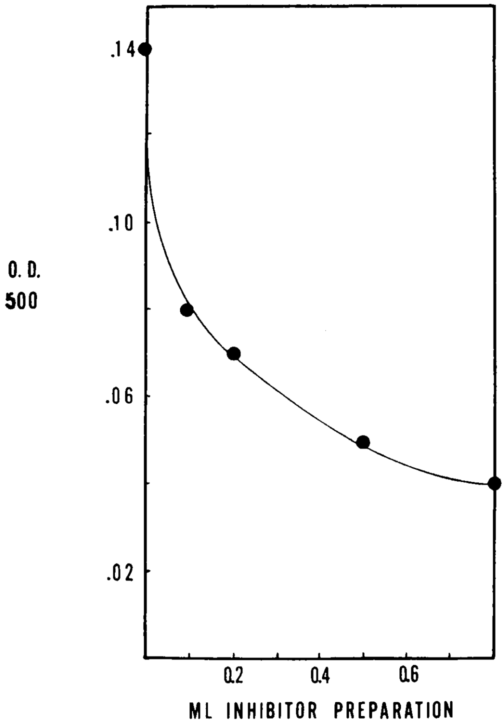

FIG. 3. Tryptic digestion of casein in the presence of inhibitor, fraction P_{IV}.

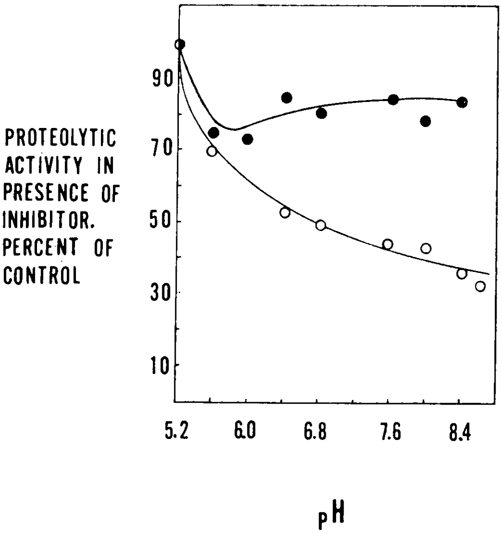

FIG. 4. The pH profile of the inhibitor of tryptic and lysosomal activities. Residual lysosomal activity (○); residual tryptic activity (●).

Summary. Inhibitors of lysosomal and tryptic hydrolysis of benzoyl-DL-arginine-β-naphthylamide (BANA) were shown to be present in the microsomal and 100,000*g* supernatant fractions of rat liver homogenates. The 100,000*g* soluble inhibitor was shown to be: (1) capable of inhibiting tryptic digestion of casein and BANA; (2) insoluble at pH 4.0 but soluble at neutral or basic pH values; (3) nondialyzable; and (4) heat-stable. The inhibitor was maximally active at pH 7.4 and above during lysosomal hydrolysis of BANA.

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