PEPSTATIN INHIBITS THE DIGESTION OF HEMOGLOBIN AND PROTEIN-POLYSACCHARIDE COMPLEX BY CATHEPSIN D*

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Summary: Pepstatin, a peptide inhibitor of pepsin isolated from cultures of Actinomycetes, is shown to be a powerful inhibitor of cathepsin D preparations obtained from bovine uterus and rabbit ear cartilage. The digestion of hemoglobin at pH 3.2 is completely blocked by pepstatin at a 1:1 molar ratio of inhibitor to enzyme. The digestion at pH 5 of protein-polysaccharide-light complex from bovine nasal cartilage is 98% inhibited.

Cathepsin D is an acid protease which resembles pepsin in its specificity and properties. This enzyme is widely distributed throughout the animal kingdom and is one of the chief enzymes of protein digestion in lysosomes (1). It is also believed to play a role in the breakdown of extracellular structures (2,3). A serious difficulty in the exploration of the biological role of this cathepsin has been the lack of a specific inhibitor that is compatible with biological systems. Inhibition has been accomplished only by the use of heavy metals (4) or by specific organic reactions with compounds such as diazoacetylnorleucine methyl ester (5). The only biologically useful inhibitor has been a specific antiserum raised against purified cathepsin D (6).

Recently, a new pepsin inhibitor, pepstatin, has been isolated from the media of cultures of Streptomyces argenteolus var. toyokaensis (7). This inhibitor has been shown to have the structure: isovaleryl-L-valyl-L-valyl-4-amino-3-hydroxy-6-methylheptanoyl-L-alanyl-4-amino-3-hydroxy-6-heptanoic acid (8). This inhibitor does not interfere with the action of a

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variety of neutral proteases, but it does strongly inhibit pepsin and several acid proteinases of microbial origin (7). It also inhibits a hemoglobin-digesting acid proteolytic activity of pig liver lysosomes, an activity believed to be due to cathepsin D (9).

The present study shows that there is, indeed, a powerful inhibition by pepstatin of cathepsin D. This is shown using a highly purified preparation of cathepsin D from bovine uterus (10) and a partially purified cathepsin D from chick embryo cartilage (11).

MATERIALS AND METHODS

Pepstatin was first brought to our attention by Dr. A. M. Dannenberg, Jr. as a potential inhibitor of cathepsin D. He kindly supplied a small amount, and more recently, a generous sample was supplied by Dr. S. Itakura of the Banyu Pharmaceutical Co., Ltd. of Tokyo, the manufacturers of pepstatin. Cathepsin D was prepared from bovine uterus (4,10) and had a specific activity of 150 units/mg. The multiple form 4 was used; this is a single chain form of molecular weight 41,000 (10). Cathepsin D was also prepared from chick embryo limb cartilage (11); it had a specific activity of 120 units/mg protein and is believed to be about 40% pure. Cathepsin D was assayed by hemoglobin digestion at pH 3.2; the assay method and definition of a unit have been described (4). The protein-polysaccharide-light (PP-L) complex was prepared from bovine nasal cartilage by the method of Pal et al. (12). Its digestion by cathepsin D was followed viscosimetrically in Cannon viscosimeters in a 25° bath. A solution of 5 ml PP-L (15mg/ml) in 0.15 M KCl was adjusted to pH 5.0, and 0.5 ml cathepsin D (1 unit) was added. The initial viscosity (in seconds flow time) was about 3 times that of water. Pepstatin was finely dispersed in saline suspension. The dilutions used for inhibition were usually sufficient to completely dissolve the inhibitor.

RESULTS AND DISCUSSION

In the first experiments (Fig. 1) hemoglobin digestion at pH 3.2

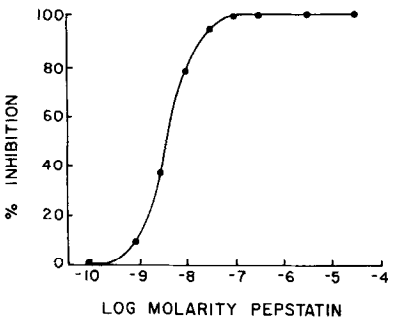

Fig. 1. Pepstatin inhibition of hemoglobin digestion by cathepsin D. The concentration of cathepsin D was 3.1×10^{-8} M.

by pure bovine cathepsin D was inhibited by the addition of pepstatin. Inhibition was 100% until the concentration of pepstatin fell below 10^{-7} M. The enzyme concentration was 3.1×10^{-8} M; at this same concentration of pepstatin, inhibition was about 15%. This indicates that there is essentially a 1:1 stoichiometry of inhibitor to enzyme. The evidence also suggests that the binding of inhibitor to enzyme is quite strong, since the 1:1 ratio inhibits almost completely. This is also borne out by dialysis experiments. Two units of enzyme were mixed with 5 mole equivalents of pepstatin in a volume of 2 ml. After standing 30 minutes at room temperature at pH 4.0 (0.05 M citrate) or pH 7.2 (0.05 M phosphate) the preparations were completely inactive against hemoglobin at pH 3.2. Dialysis overnight in the cold against 1000 volumes of these buffers restored less than 10% of the activity of the pH sample, and no activity at pH 7.2. Samples of enzyme carried through these steps without added pepstatin served as controls.

The results agree well with the results obtained with pepsin. Aoyagi et al. (13) found a 50% inhibition of hemoglobin digestion when the pepstatin concentration was 1/3 the pepsin concentration and almost complete inhibition when the inhibitor concentration was equal to the pepsin concentration. They also found that the binding was quite tight ($K_i < 3 \times 10^{-9}$ M). The binding is due chiefly to apolar bonding and to hydrogen bonding to the hydroxyl group on the carboxyl-terminal residue. Binding was reversed by

raising the pH to 7, but the pepsin was destroyed. My experiments at pH 7.2 do not distinguish between reversal of binding with enzyme destruction or irreversibility of binding.

Ikezawa et al. (9) found that lysosomal digestion of hemoglobin at pH 3.2, probably attributable to cathepsin D, was 50% inhibited by 1.5×10^{-8} M pepstatin. It is not possible to calculate the exact concentration of cathepsin D in this crude preparation, but the result seems to be in reasonably close agreement with the present results on pure enzyme.

In the second group of experiments, cathepsin D was used to digest PP-L complex. It has been found that cartilage contains cathepsin D (2,11), and experiments on the breakdown of cartilage matrix by ruptured lysosomes have demonstrated that the breakdown can be blocked by specific antisera to cathepsin D (3). An inhibitor of cathepsin D might assume considerable importance in the treatment of cartilage disorders such as osteoarthritis.

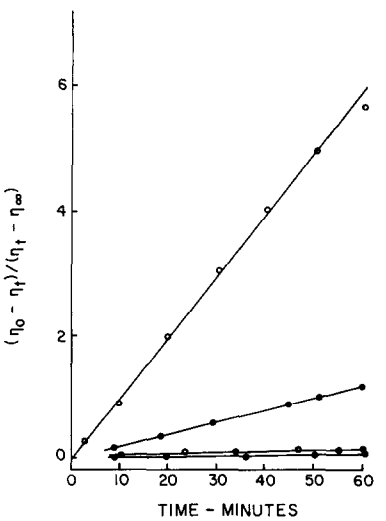

Fig. 2. Digestion of protein-polysaccharide-light complex by cathepsin D from bovine uterus (o) and chick limb cartilage (●). The two lowest lines represent the activity in the presence of pepstatin, 2.5×10^{-7} M. The initial viscosity (η_o) minus the viscosity of completely digested PP-L (η_∞) was taken as the total viscosity change. Second order kinetics were observed, that is, the viscosity change by time t ($\eta_o - \eta_t$) divided by the viscosity change yet to occur ($\eta_t - \eta_\infty$) gave a linear plot against time.

As seen in Fig. 2, bovine cathepsin D rapidly digests PP-L; 1 unit causes an 85% reduction in viscosity in 1 hour at pH 5.0 and 25°. One unit of chick cartilage cathepsin D, defined by the hemoglobin assay, is only about 1/5 as active in degrading PP-L as the uterus enzyme. However, pure chicken cathepsin D has twice the specific activity of mammalian cathepsin D on hemoglobin (14), so its active center must differ from that of the uterus enzyme. Both the cow and the chick enzyme are inhibited to the extent of 98% by the addition of 1 μg pepstatin. This corresponds to a ten-fold molar excess of pepstatin. There is a slow spontaneous change in the viscosity of PP-L which makes it difficult to determine whether the small activity seen in the presence of pepstatin is actually significantly greater than the blank. In any event, the digestion of PP-L at pH 5 can be attributed almost exclusively to the action of cathepsin D, even in the impure preparation of the cartilage enzyme.

The effectiveness of pepstatin in inhibiting cathepsin D over the pH range 3-5 suggests an important use for this compound in biological studies of the role of cathepsin D. Umezawa et al. (7) have shown that this material has a very low level of toxicity when administered orally or intraperitoneally in various common laboratory animals; it also inhibits rat paw edema induced by carrageenin. Recently, Gross et al. (15) showed a direct inhibition of renin action in vivo in rats using intravenous injections of pepstatin. These results suggest the possibility of using pepstatin to ascertain the role of cathepsin D in situations such as intracellular digestion of proteins, extracellular matrix digestion accompanying lysosome rupture, and experimental models of arthritis. The present results also serve to underline the close relationship between cathepsin D and pepsin.

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