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CONCENTRATION AND TEMPERATURE EFFECTS ON OVOSTATIN ACTIVITY

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Introduction

Ovostatin is a 780,000 MW protein, originally isolated from chicken egg white (1). Structural studies indicate that the protein is a tetramer of identical subunits of 165,000 MW which can be separated upon reduction with β-mercaptoethanol. Chicken ovostatin exhibits protease inhibitor activity against metalloproteases such as collagenase and thermolysin (1), and of acid proteases such as pepsin and rennin (2). Ovostatin isolated from duck eggs (3) and crocodile eggs (4) appears to be similar to chicken egg ovostatin, but with significant differences in structure and function. Duck ovostatin contains a reactive thiol ester which is not found in the chicken protein, and duck and crocodile ovostatin inhibit serine proteases such as trypsin and chymotrypsin, while the chicken protein does not. Electron microscopy (4,5) of ovostatin indicates that two subunits associate near the middle of each polypeptide to form a dimer with four arms. Two of these dimers then associate to produce a tetramer with eight arms, with the protease binding site near the center of the molecule. Upon binding of the protease, the enzyme cleaves a susceptible bond in the "bait" region of the ovostatin. Cleavage of this bond causes a conformational change in which all eight arms of the ovostatin molecule curl up towards the center, effectively trapping the protease and sterically hindering access of large substrates to its active site. The structural organization and mechanism of action proposed for ovostatin are nearly identical to that proposed for α_2-macroglobulin, a serum protease inhibitor (6) which may play an important role in regulation of proteases in animal tissues.

Although the general arrangement of subunits appears to be the same for all ovostatins studied so far, some differences have been observed, with chicken ovostatin more closely resembling reptilian ovostatin than the duck protein. This is a surprising result, given the evolutionary relatedness of chickens and ducks. It is possible that the difference in structures may be due to deformed subunit arrangements which occur during the processing and fixing necessary for electron microscopy (4). Examination of the native structures of these proteins using X-ray crystallography would help clarify these discrepancies. Crystals of chicken ovostatin have recently been prepared at MSFC which will allow such studies to be performed.

Body

Previous experiments using the chicken ovostatin purified at MSFC indicated that it did not display the same degree of inhibitory activity against thermolysin as had been reported by Nagase *et al.* (1). Using azocasein as a substrate for thermolysin, Nagase found that an ovostatin/thermolysin molar ratio (O/T) of 0.5 produced 50% inhibition, and a ratio of 1.0 produced 90% inhibition. Based on these results they concluded that the stoichiometry of interaction between ovostatin and thermolysin was 1:1. Assay of the MSFC ovostatin indicated that a much higher ratio of ovostatin to thermolysin was needed to produce a similar level of inhibition. For example, an O/T of 2.0 produced only about 50% inhibition at 23°C, and an O/T greater than 8.0 was required to produce nearly 90% inhibition. Experiments were therefore undertaken to try to understand properties of the ovostatin or factors in the assay which could produce these results. The ovostatin purification scheme was examined by assaying the various column fractions at different steps in the procedure. It was found that the thermolysin inhibitory activity coincided with the protein that was being purified and that the

ovostatin did not appear to be losing activity through the purification process. The purified ovostatin preparations have been used to successfully prepare crystals of the protein.

Light scattering experiments performed by Dr. William Wilson at Mississippi State University using the MSFC ovostatin preparations indicated that at low ovostatin concentrations, below 0.2 mg/ml, the protein was dissociating from a tetramer into dimers. Since the proposed mechanism of action involved the tetrameric form of the protein, we hypothesized that perhaps under the conditions of our assays at various O/T ratios the ovostatin was becoming dissociated into an inactive dimer. To examine this possibility we assayed the ovostatin activity as a function of ovostatin concentration and of temperature of the assay. Figures 1-4 below show the results of these assays at 23°C, 30°C, 37°C and 42°C respectively.

Fig. 1: Effect of [OVSTN] at 23°C

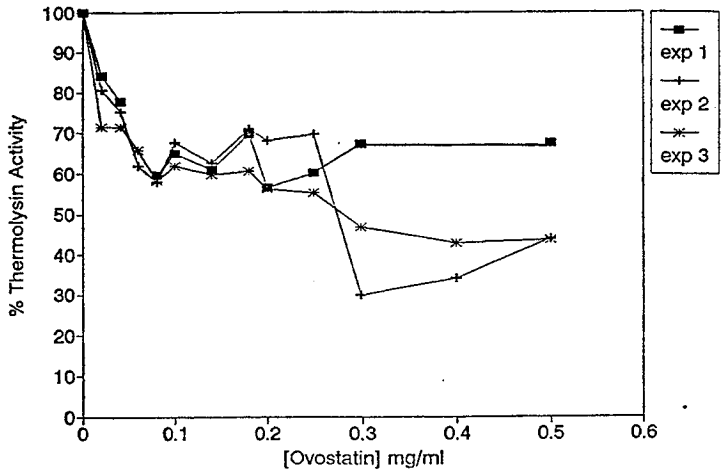


Fig. 2: Effect of [OVSTN] at 30°C

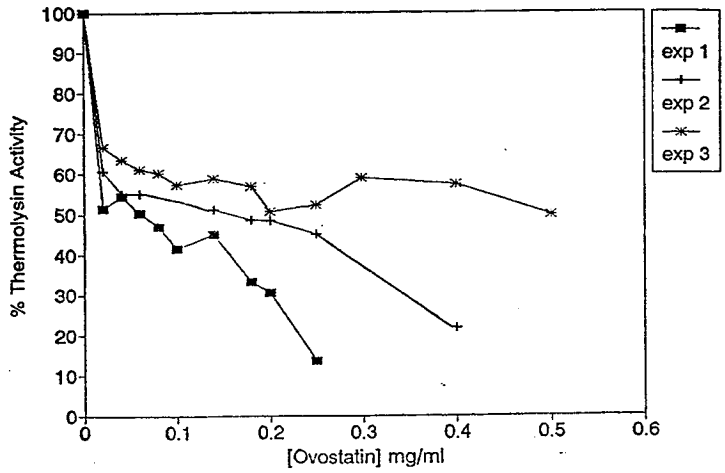


Fig. 3: Effect of [OVSTN] at 37°C

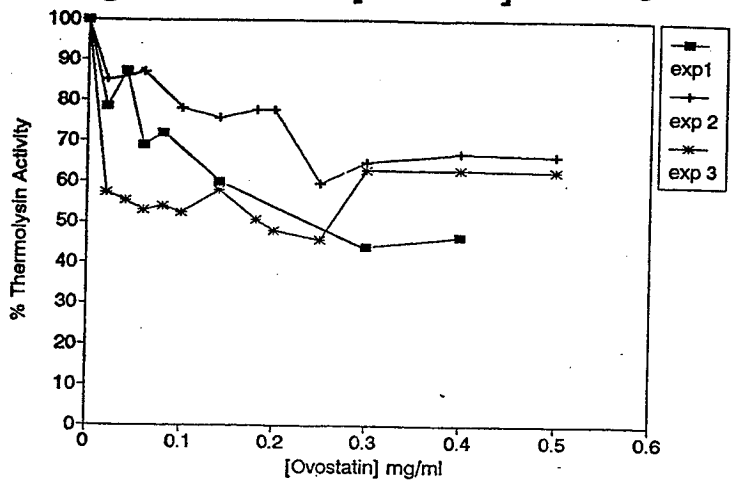
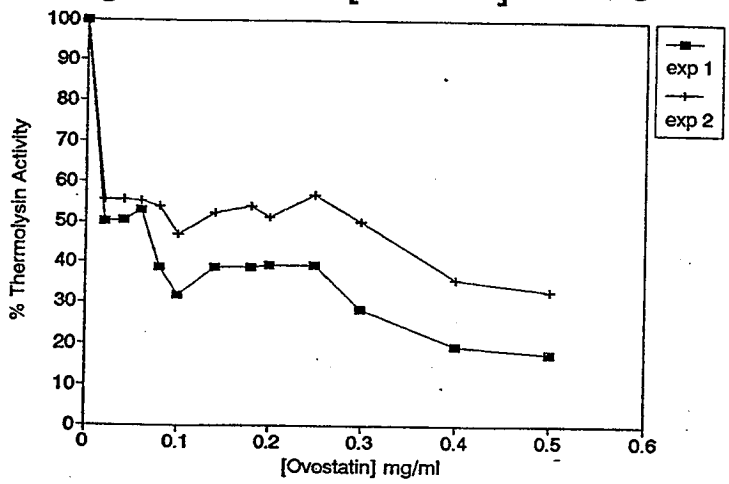


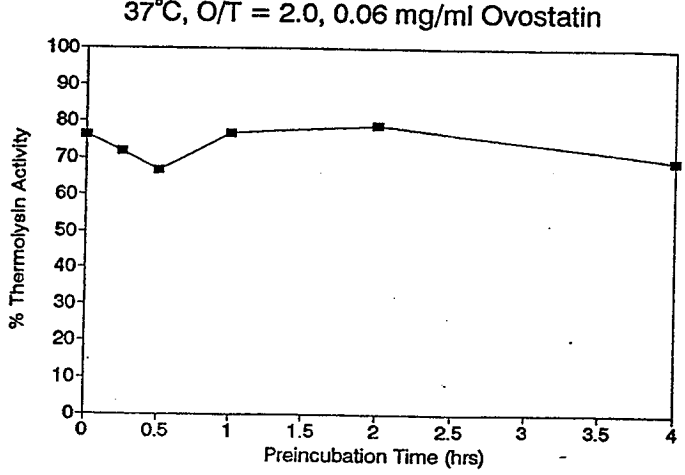
Fig. 4: Effect of [OVSTN] at 42°C



The data at 23°C clearly indicate an increase in activity of the ovostatin going from 0.2 to 0.8 mg/ml. The data at the other temperatures is less clear, but does show a trend towards less activity of ovostatin at lower concentrations, below 0.06 mg/ml. However, at concentrations where the light scattering data indicate that the ovostatin exists primarily in the dimer, and supposedly less active, form, we still observe between 20 and 50% inhibition at the different temperatures. Thus, it is possible that the dimer form of the ovostatin may also be able to interact with the thermolysin in such a way as to inhibit it. The data also appears to indicate that there is not a significant affect of temperature on the ovostatin, although there may be an observable difference between the 23°C data and the 42°C data, with slightly higher ovostatin activity at the higher temperature. These results are consistent with recent light scattering data which also showed no significant affect of temperature on the tetramerdimer dissociation.

In considering the light scattering data showing the dissociation of the ovostatin, we questioned whether there is a time dependence of the dissociation, reflected in lower activity of the dimer with increased time a the lower concentrations. We therefore, diluted the ovostatin to 0.06 mg/ml, a concentration at which the ovostatin should exist primarily as the dimer, and incubated it at 37°C for various times from 15 minutes to 4 hours, before adding the thermolysin. Figure 5 shows that there was no significant difference in the ovostatin activity over this time interval.

Fig.5: Effect of Time on OVSTN Activity



During the course of these studies it was noted that Nagase *et al.* (1) had not reported the source of the thermolysin used in their assays. A search of the commercially available thermolysin preparations revealed that two different specific activity preparations are available: a lower specific activity thermolysin from Sigma Chemical Co., which is the one we have been using, and a higher specific activity product from Calbiochem. Since part of the proposed mechanism of action for ovostatin inhibition of thermolysin involves cleavage of a peptide bond in the ovostatin by the thermolysin, the activity of the enzyme itself could conceivably affect the observed inhibitory activity of the ovostatin. We attempted to obtain this higher specific activity thermolysin, but were told that it was backordered until late August, 1994. Thus, it will not be possible to test the hypothesis that the thermolysin itself is causing the discrepancy between our results and those of Nagase *et al.* (1) until after this program has been terminated.

Conclusions

The data are highly suggestive that there is a decrease in ovostatin activity as the concentration of the protein falls below 0.06 mg/ml. This may not be of any physiological importance, however, since the concentration of ovostatin in the egg is about 0.5 mg/ml. Curiously, the dissociation of the tetramer into dimers does not show a significant temperature dependence as would be expected for an equilibrium reaction. Whether this is in fact the case, or whether the differences are so small as to not be discerned from the current data remains to be seen. Another aspect to consider, is that in the egg the primary role of the ovostatin may or may not be as a protease inhibitor. Although the inhibition of collagenase by ovostatin may be an important aspect of embryogenesis, it is also possible that it functions as a binding protein for some substance. In this regard, all ovostatin preparations from MSFC have shown an approximately 88,000 MW protein associated with the ovostatin. The identity of this protein is not currently known and may be the subject of future studies.

References

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