ABSTRACT

Human blood contains several inhibitors of proteolytic enzymes which are implicated in the regulation of physiological processes such as blood coagulation, complement fixation and response to inflammation. These inhibitors exert their regulatory functions by maintaining a proper proteinase-proteinase inhibitor equilibrium in the tissue. Alpha_1-antichymotrypsin is one of the important human plasma/serum inhibitor which inhibits serine proteinases. In view of the desirability of getting relevant informations on alpha_1-antichymotrypsin from mammals other than human and the fact that inhibitor was found to be absent in certain mammalian serum e.g. porcine, it was thought worthwhile to isolate and characterize the inhibitor from hitherto uninvestigated source i.e. goat.

Goat chymotrypsin inhibitor was isolated both from serum and plasma and their properties were found to be indistinguishable. Goat plasma showing 5% inhibitory activity per milligram of protein was salt fractionated and further purified by ion exchange chromatography on DEAE-cellulose column (2.4 x 6.0 cm) equilibrated with 10 mM sodium phosphate buffer, pH 7.5, containing 50 mM NaCl and 0.02% sodium azide. The protein was eluted
batchwise with the buffer containing varying concentrations of sodium chloride (0.1 M - 0.2M NaCl). The protein fractions eluted with the buffer containing 150 mM sodium chloride, showing optimal antichymotryptic activity, were pooled and rechromatographed on the same ion exchange column. The final protein yield of the inhibitor was 0.15% (W/W). Thus about 80 mg of the pure inhibitor was isolated from the goat plasma containing 53 g of protein by the procedure used in this study.

The goat inhibitor preparation moved essentially as a single protein band in 12% polyacrylamide gel during electrophoresis performed in Tris glycine buffer (25 mM Tris and 194 mM glycine), pH 8.3, containing 0.1% SDS with a relative mobility of 0.26. This showed that the preparation of goat chymotrypsin inhibitor obtained in this study was homogeneous with respect to size. The size homogeneity was also indicated by gel filtration on Sephadex G-200 column to be described below.

When the measured relative mobility (0.26) of the chymotrypsin inhibitor was compared with the relative mobilities of marker proteins viz, transferrin, bovine...
serum albumin, Ig G, ovalbumin, chymotrypsinogen A and cytochrome c which were electrophoresed under conditions identical to that used for the electrophoresis of the inhibitor, the molecular weight of the inhibitor was found to be 68 kDa. The observation that relative mobility of the chymotrypsin inhibitor remained unaltered under reducing (0.02 M 2-mercaptoethanol) and nonreducing conditions taken together with the conclusion that the native molecular weight (76 kDa) as determined by gel filtration suggested that the inhibitor consisted of a single polypeptide chain. The native molecular weight (76 kDa) as determined by gel filtration under native condition is about 12% higher than that (68 kDa) determined by polyacrylamide gel electrophoresis under denaturing conditions. The discrepancy has been ascribed to the glycoprotein nature of the inhibitor.

Goat chymotrypsin inhibitor was found to be a sialoglycoprotein containing 4.5% sialic acid and 12% neutral hexose; these results would mean that the inhibitor molecule contains 10 moles of sialic acid and 47 moles of neutral hexose.
The number of free sulfhydryl group in the goat chymotrypsin inhibitor was determined by titration with p-hydroxymercuribenzoate in 10 mM sodium phosphate buffer, pH 7.0. From the curve between increase in absorbance at 250 nm and molar ratio of p-hydroxymercuribenzoate to inhibitor, the number of free sulfhydryl group per mole of inhibitor was calculated as one. Interestingly, the goat chymotrypsin inhibitor with sulfhydryl group blocked with p-hydroxymercuribenzoate retained its full antichymotryptic activity suggesting that the lone titrable sulfhydryl group in the inhibitor had no functional role.

The isoionic preparation of goat chymotrypsin inhibitor was prepared by passing extensively dialyzed inhibitor solution through Dintzis column. The pH of the inhibitor solution containing 1.6 mg/ml protein was determined to be 5.5. The concentration of isoionic inhibitor solution was measured by dry weight method. The goat inhibitor solution (0.11 mg/ml) absorbed maximally at 278 nm in 10 mM sodium phosphate buffer, pH 7.5, containing 150 mM sodium chloride. Fluorescence excitation and emission spectra of the goat inhibitor were obtained in the same buffer but at relatively lower protein concentration (0.02 mg/ml). The emission and
and excitation maxima were found to occur near 338 and 278 nm, respectively. These spectral features suggest that inhibitor is a tryptophan containing protein.

The specific extinction coefficient of goat chymotrypsin inhibitor was computed from the measured optical densities of protein solutions of known concentrations. The values of specific extinction coefficient were found to be 6.23 and 5.92 cm$^2$g$^{-1}$ respectively at 278 and 280 nm.

The hydrodynamic properties of chymotrypsin inhibitor were studied by analytical gel chromatography on a Sephadex G-200 column (2.4 x 78 cm) equilibrated with 10 mM sodium phosphate buffer, pH 7.5, containing 150 mM sodium chloride and 0.02% sodium azide. The column was calibrated with marker proteins of known molecular weights and Stokes radii. These were bovine serum albumin, ovalbumin, pepsin, chymotrypsinogen A and cytochrome c. The elution volume of the goat chymotrypsin inhibitor from the column was measured to be 202 ml which would correspond to a molecular weight of 76 kDa and Stokes radius of 3.53 nm. The Stokes radius of the inhibitor was used in the calculation of its diffusion coefficient and frictional ratio which were found to be $6.29 \times 10^{-7}$
cm²/sec and 1.32, respectively. Thus the measured frictional ratio (1.32) is significantly different than that expected for a globular protein. Alternatively this may be due to unusually high degree of hydration of the goat sialoglycoprotein chymotrypsin inhibitor containing as high as 17% carbohydrate.

The thermal stability of the goat chymotrypsin inhibitor was studied in 10 mM sodium phosphate buffer, pH 7.5, containing 150 mM sodium chloride in the temperature range, 37°C-60°C. The inhibitor was kept at a given temperature for 15 minutes and its inhibitory activity was measured against bovine chymotrypsin at 37°C. The inhibitor incubated at 37°C and 45°C showed the same inhibitory activity. However, by treatment of the inhibitor at 50°C and 55°C, the losses in inhibitory activity were 35% and 65% respectively. When the inhibitor was incubated at 60°C for 15 minutes and its inhibitory activity was measured, no activity was detected suggesting the inactivation of the inhibitor at 60°C.

Unlike human alpha₁-antichymotrypsin the goat chymotrypsin inhibitor was able to inhibit even trypsin
at relatively higher inhibitor concentration. Thus under identical conditions complete inactivation of chymotrypsin was achieved at inhibitor to enzyme molar ratio of 1.1. However, with trypsin this ratio increased to 4.5. For time course studies chymotrypsin and trypsin were treated with the goat chymotrypsin inhibitor at a molar ratio ([I]/[E]) of 1.1 and 5.0 respectively in 10 mM sodium phosphate buffer, pH 7.5. containing 150 mM sodium chloride. The residual caseinolytic activity of the mixture was measured at different time intervals. The inhibition of chymotrypsin was completed within 5 minutes, however, as much as 50 minutes were required to achieve complete inhibition of the caseinolytic activity of trypsin. This observation suggests that the goat chymotrypsin inhibitor inhibited chymotrypsin in a biologically feasible time while inhibition of trypsin by the inhibitor appeared to have little or no biological significance. From the time course of inactivation of chymotrypsin by the goat inhibitor, the rate constant for the association of the inhibitor with the enzyme was calculated to be $1.6 \times 10^{-4} \text{ M}^{-1} \text{ s}^{-1}$ at $37^\circ\text{C}$ which is comparable to that found for human inhibitor - chymotrypsin system.