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DIFFERENTIAL AUTOLYSIS OF HUMAN AND CANINE PLASMINS

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SUMMARY

Plasmin, like trypsin, undergoes autolysis. The proteolytic activity of human plasmin was virtually abolished after incubation at 37° for 1 hour, whereas the activity of the canine plasmin remained relatively stable under the same conditions. After 23 hours of incubation at 25°, canine plasmin had lost only 12% of its proteolytic activity, while human plasmin had lost 72% of its activity. Gel electrophoretic analyses showed differences in the susceptibility to proteolysis of the light and heavy chain components of the two plasmin molecules. The light chain of canine plasmin was relatively stable to autolysis while the light chain of human plasmin underwent extensive proteolytic degradation. The patterns of digestion of the heavy chain components of the two plasmin species were also found to be different. The results obtained in this study suggest that significant differences exist in the structures of human and canine plasmins.

The fibrinolytic enzyme plasmin (EC 3.4.4.14) is present in blood as the inactive zymogen, plasminogen. Plasminogen is activated to plasmin by cleavage of an arginyl-valine peptide bond; as a result the single chain plasminogen molecule becomes converted to the two-chain structure of plasmin (1). The enzymatic active site is present in the light chain of the molecule. In human plasmin the heavy chain of 57,000 daltons is connected to the light chain of 26,000 daltons by two disulfide bridges (2,3). Plasminogen can be activated to plasmin by the addition of the bacterial protein streptokinase. However, streptokinase does not have the ability to cleave peptide bonds in proteins. The mechanism by which streptokinase activates plasminogen has been described recently (4,5). Streptokinase reacts with plasminogen by forming a stoichiometric complex. As a result of this complex formation, an active center develops in the plasminogen moiety which catalyzes the cleavage of the activation peptide bond in the plasminogen molecule.

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Significant differences have been found to exist in the interaction of streptokinase with human and dog plasminogens (6,7). The activation of dog plasminogen, in contrast to human plasminogen, is dependent on the concentration of streptokinase used for activation. Moreover, the rate of active site formation in the dog plasminogen is relatively slow; in an equimolar mixture of streptokinase and dog plasminogen more than 10 minutes are required for the formation of 1 mol of active site (7). In the human system active site formation occurs within 15 seconds under identical conditions. It was of interest to determine if the differences in the human and canine plasminogen molecules were also present in the active enzyme species. In this study we have used proteolytic susceptibility as a probe for studying the human and canine plasmin molecules. Plasmin, like trypsin, is highly specific in cleaving arginine and lysine peptide bonds. The human and canine enzymes were allowed to undergo autolysis and the resultant changes in the respective protein molecules were determined.

MATERIALS AND METHODS

Human and dog plasminogens were purified by the affinity chromatographic method of Deutsch and Mertz (8) as described previously (5,7). Out-dated human plasma was obtained from the local blood bank. Dog plasma was purchased from Pel-Freez Biologicals, Inc. Urokinase was purchased from Calbiochem Corp. Streptokinase was a generous gift of Hoechst Pharmaceutical Company. Para-nitrophenyl-p-guanidinobenzoate HCl (NPGB)[1] was obtained from ICN Pharmaceuticals, Inc.

Human and canine plasmins were prepared by activating the plasminogens with catalytic amounts of urokinase (500 units per mg. of plasminogen), in 0.1M Pipes buffer, pH 7.2. The opalescent activation mixtures were clarified by centrifugation. Human plasmin was also prepared by activation with catalytic amount of streptokinase (600 units per mg. of plasminogen). Plasmin activity was determined using azocasein as substrate (5). Acrylamide gel electrophoresis in sodium dodecyl sulfate was carried out according to the method of Weber and Osborne (9) using 7.5% gels. Quantitative determination of the heavy and light chain degradation was done by integration of the area under each peak of the gel scan tracing.

Autolysis of human and canine plasmins. Human and canine plasmin preparations in 0.1M Pipes buffer, pH 7.2 (0.62 mg/ml) were incubated at the indicated temperature. At various time intervals, aliquots from the reaction mixture were analyzed for enzyme activity by the azocasein method. Structural changes in the heavy and light chains of plasmin were detected by SDS gel electrophoretic analyses after reducing the protein samples with mercaptoethanol. The active site of canine plasmin was blocked by reaction with a 100-

[1] The abbreviations used are: NPGB, p-nitrophenyl-p'-guanidinobenzoate; Pipes, piperazine-N,N'-bis(2-ethanesulfonic acid); SDS, sodium dodecyl sulfate.

fold excess of NPGB. After removing the excess reagent by dialysis the protein preparation was subjected to proteolysis by adding 25% (w/w) human plasmin.

RESULTS AND DISCUSSION

Figure 1 shows the changes in proteolytic activity of human and canine plasmins incubated at 37°. Human plasmin lost enzyme activity very rapidly, 86% in one hour. Identical results were obtained with both urokinase and streptokinase activated human plasmin preparations. Canine plasmin lost only 12% of its proteolytic activity after 1 hour of incubation. In 3 hours when human plasmin lost virtually all of its enzyme activity, canine plasmin still retained 80% of its biological activity. SDS-gel electrophoretic analyses of aliquots of plasmin taken at various time intervals indicated that proteolytic hydrolysis of peptide bonds in both heavy and light chain components had occurred. To assess these changes in greater detail, studies of the autolysis of human and canine plasmins were carried out at 25°. Figure 2 shows the expected decrease in the loss of proteolytic activity of the plasmin samples incubated at 25°. Nevertheless, it can be seen that human plasmin progressively lost its activity, with only 28% of the initial biological activity remaining at the end of a 23 hour incubation period. Canine plasmin on the other hand retained 88% of its initial activity in the same time interval.

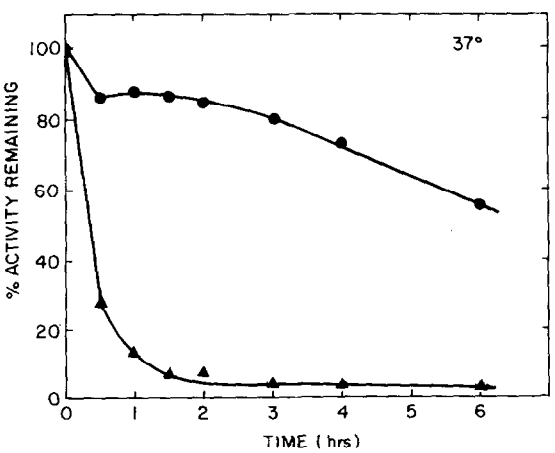

Fig. 1. Time course of autolysis of plasmin samples incubated at 37° in 0.1M pipes buffer, pH 7.2. Plasmin activity remaining at the end of the indicated time intervals was determined by azocasein digestion. (▲—▲ human plasmin), (●—● canine plasmin).

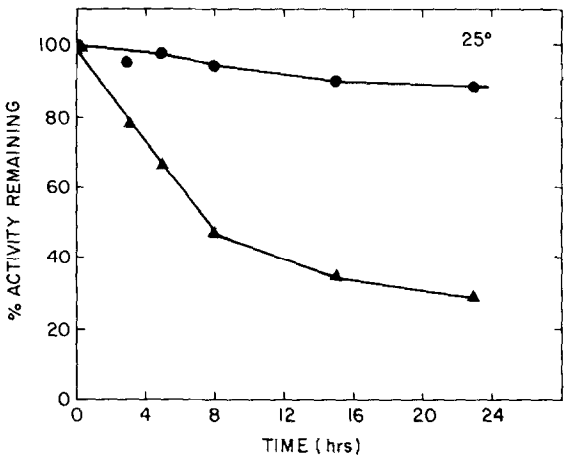

Fig. 2. Autolysis of human plasmin (▲—▲) and canine plasmin (●—●) incubated at 25° in 0.1M pipes buffer, pH 7.2.

Aliquots from the plasmin reaction mixture undergoing autolysis at 25° were analyzed by SDS-gel electrophoresis. Figure 3A shows the densitometric tracings of the gel pattern obtained in the human system, and figure 3B shows

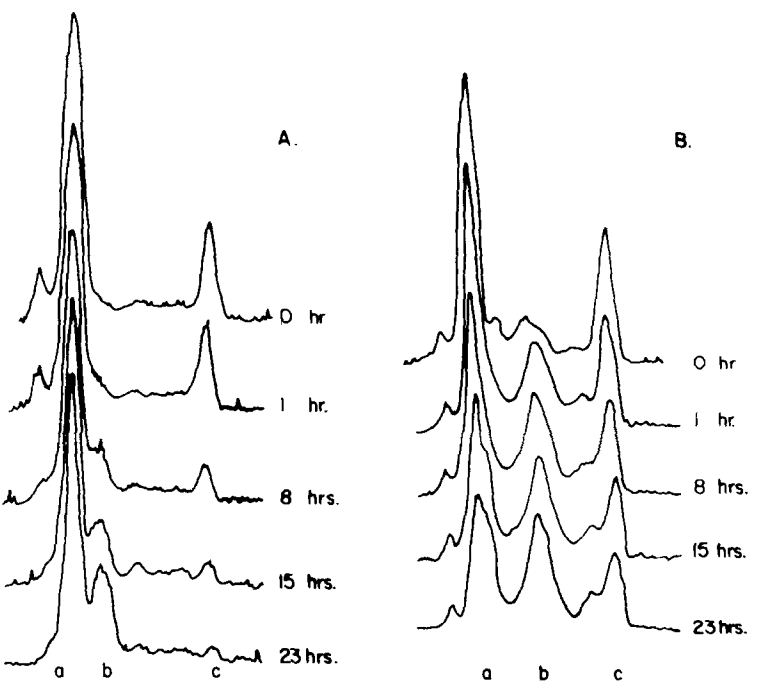

Fig. 3. Tracings of the SDS-gel electrophoretic patterns of autolysed products obtained from human plasmin(A) and from canine plasmin(B). The plasmin samples were incubated at 25° and aliquots taken for electrophoresis at the indicated times. Peak (a) represents the heavy chain of plasmin, peak (b) is the main degradation product of the heavy chain, and peak (c) is the light chain of plasmin.

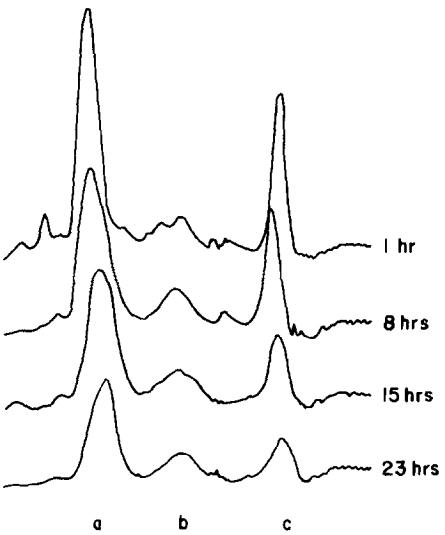

Fig. 4. Tracings of the SDS-gel patterns of degradation products obtained by digesting NPGB-reacted canine plasmin with 25% (w/w) human plasmin. Peaks a, b, and c are as in fig. 3. For details see text.

the tracings from the canine system. It can be seen that significant differences exist in the degree of proteolysis of the heavy and light chain components of the two plasmin species. In human plasmin the light chain component was found to undergo progressive degradation during the course of incubation. Measurement of the peak area representing the light chain of human plasmin after various time intervals of autolysis gave the following results: after 5 hours 66% of the initial amount of the light chain was present, and 44%, 31%, and 23% of the light chain were found after 8, 15, and 23 hours of incubation respectively. This loss in the light chain component corresponds very well with the loss in enzyme activity, in agreement with the results reported by Gaffney et. al. (10). The heavy chain component remained relatively intact, 90% of the initial amount being present after 23 hours of incubation. In the dog system, the light chain was relatively stable to proteolysis, 91% of the light chain was present after 8 hours of incubation at 25°. After 23 hours of incubation 77% of the light chain was present intact. This stability of the light chain in canine plasmin correlates very well with the observed stability of its biological activity. Significant differences have also been found in the proteolysis of the heavy chain component of the

two plasmin species. The heavy chain of canine plasmin was found to be degraded to a greater extent than the heavy chain of human plasmin. Moreover, the pattern of digestion was different. While the autolysis of human plasmin gave rise to a 50,000 dalton fragment of the heavy chain as one of the major products, in the dog a much smaller size fragment migrating between the heavy and light chains (band C, fig. 3B) was present.

The relative stability of the light chain of canine plasmin may indicate the inaccessibility of proteolytically susceptible peptide bonds in this region of the plasmin molecule. To further explore this possibility, the following experiment was performed. The active site of canine plasmin was blocked by reaction with NPGB to prevent its autolysis during the course of the experiment. This preparation was subjected to proteolysis using human plasmin. Figure 4 shows the tracing of the gel electrophoretic patterns obtained. It can be seen that the light chain of canine plasmin had also undergone proteolysis indicating that this chain does contain peptide bonds susceptible to proteolysis. In a control experiment when active site-blocked dog plasmin was digested with dog plasmin, the results obtained were similar to those seen in fig. 3B, i.e. the light chain remained intact. This showed that the blocking of the active site in dog plasmin had no significant effect on the conformation of this protein molecule.

The determination of the complete amino acid sequence of human plasminogen has been reported recently (2,3) while the sequence of canine plasminogen is not known. The results obtained in this study show that human and canine plasmins may have differences in their amino acid sequences which are reflected in differences in the conformation of their light chain regions. These differences in human and canine plasmins could account for the differences observed in the interaction of streptokinase with these two plasmin species (7) since streptokinase is known to bind to the light chain of human plasmin (11). Recently Christensen et. al. (12) have shown that the enzyme activity of a human 'miniplasmin' preparation was quite similar to that of

native plasmin. This miniplasmin had an intact light chain and contained a modified heavy chain of 110 amino acid residues. The heavy chain of native plasmin contains 560 residues. In the present study we found that when dog plasmin undergoes proteolysis in its heavy chain, the modified plasmin obtained was also enzymatically active. The isolation and characterization of this modified canine plasmin is in progress.

ACKNOWLEDGEMENT

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REFERENCES

1. Robbins, K.C., Summaria, L., Hsieh, B., and Shah, R.J. (1967) J. Biol. Chem. 242, 2333-2342.
2. Sottrup-Jensen, L., Claeys, H., Zajdel, M., Petersen, T.E., and Magnusson, S. (1977) Progress in Chemical Fibrinolysis and Thrombolysis, Vol. 3, pp. 191-209, Raven Press, New York.
3. Wiman, B. (1978) Fibrinolysis, pp. 47-60, Academic Press, New York.
4. McClintock, D.K., and Bell, P.H. (1971) Biochem. Biophys. Res. Commun. 43, 694-702.
5. Reddy, K.N.N., and Markus, G. (1972) J. Biol. Chem. 247, 1683-1691.
6. Summaria, L., Arzadon, L., Bernabe, P., and Robbins, K.C. (1974) J. Biol. Chem. 249, 4760-4769.
7. Reddy, K.N.N. (1976) J. Biol. Chem. 251, 6624-6629.
8. Deutsch, D.G., and Mertz, E.T. (1970) Science, 170, 1095-1096.
9. Weber, K., and Osborn, M. (1969) J. Biol. Chem. 244, 4406-4412.
10. Gaffney, P.J., Brasher, M., Lord, K., and Kirkwood, T.B.L. (1977) Haemostasis 6, 72-88.
11. Summaria, L., and Robbins, K.C. (1976) J. Biol. Chem. 251, 5810-5813.
12. Christensen, U., Sottrup-Jensen, L., Magnusson, S., Petersen, T.E., and Clemmensen, I. (1979) Biochem. Biophys. Acta. 567, 472-481.