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FRACTIONATION AND PARTIAL CHARACTERIZATION OF α-1-PROTEASE ISOINHIBITORS OF HORSE

ANTONIO PELLEGRINI and ROLAND VON FELLENBERG

Department of Veterinary Physiology, University of Zürich, Winterthurerstrasse 260, 8057 Zürich (Switzerland)

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Summary

The principal α-1-protease inhibitor of horse was fractionated by classical methods and analysed with a modified fibrinogen-agarose gel electrophoretic method of high sensitivity and resolving power. Starting with an electrophoretically homogeneous inhibitor in unfractionated serum, two isoinhibitor bands became apparent after fractionation with $(NH_4)_2SO_4$ and DEAE-cellulose DE-52 ion-exchange chromatography. The isoinhibitors differed in electrophoretic migration and in the elution pattern from Sephadex G-100 gel filtration, but possessed identical antigenic determinants and enzyme specificity. The slower migrating isoinhibitor with an apparent molecular weight of 90 000 could be highly purified. In contrast the faster moving isoinhibitor (molecular weight 65 000) could not be completely freed from a contaminating α-2-protease inhibitor. The formation of the two isoinhibitors is discussed considering conformational changes analogous to phenomena observed with α-2-macroglobulin, or dimer formation in combination with altered conformations. The isoinhibitors described here are new additions to the different heterogeneities which exist in α-1-protease inhibitors in horse. They also supplement the different heterogeneities which exist among the α-1-protease inhibitors of mammals.

Introduction

Since the detection of genetic α-1-antitrypsin deficiency in man [1,2] and its link to the early onset of panlobular emphysema [3], a dysequilibrium between proteases and their inhibitors has been implicated in the pathogenesis of chronic-obstructive lung disease [4].

The horse is the only well-managed animal species which suffers spontaneously from chronic-obstructive lung disease in a similar way to man [5]. In addition the horse has a similar submacroscopic lung anatomy to the human [6]. As a matter of fact chronic-obstructive lung disease is the most frequent internal disease observed in the horse [7]. Despite the fact that genetic α-1-protease inhibitor deficiency has not been related to emphysema in the horse, it is the ideal animal for experimental and clinical emphysema research.

For these reasons we have started to analyze the protease inhibitors of horse in the hope that peculiarities of the protease inhibitors with respect to their content, distribution or structure may contribute to the understanding of the pathogenesis of lung emphysema. First results with the serum inhibitors have revealed that the α-1-protease inhibitors are heterogeneous, when analysed by a modified fibrinogen-agarose gel electrophoresis of high resolution and sensitivity at pH 8.6 [8,9]. In addition individual differences were observed concerning the number and electrophoretic position of the α-1-protease inhibitors. Some horses had one, others had two α-1-protease inhibitor bands. Thus, the horses could be grouped according to their pattern of inhibitors. For some individual horses the inhibitory patterns for trypsin, chymotrypsin and elastase were divergent, suggesting that multiple inhibitors with differing enzyme specificity existed. So we strongly feel that only fractionation and purification of the inhibitors would help us to clarify and understand this complex system and its physiological meaning and importance. As a first result of these efforts we report in this communication an additional type of heterogeneity of the α-1-protease inhibitor system of the horse, which only became apparent as the result of biochemical fractionation.

Material and Methods

Animals. As already mentioned in the introduction there are individuals among the horse population which have only a single α-1-protease inhibitor in the unfractionated serum [8]. Such a horse was selected as blood donor.

New Zealand white rabbits used for antiserum production were obtained from the animal breeding facilities of the Tierspital, Zürich.

Material. 1-*o*-methyl-α-D-glucopyranoside was from Fluka. *N*-Benzoyl-arginine-*p*-nitroanilide, horse hemoglobin, crystalline bovine serum albumin, pancreatic elastase, bovine chymotrypsin and Bromophenol blue were from Serva. Trypsin (Trypure®) and plasmin (Lysofibrine®) were from Novo Industri. Bovine fibrinogen was from Calbiochem. Agarose was from Seakem. Con A-Sepharose, Sepharose 4B, Sephadex G-100 and the gelfiltration calibration kit for molecular weight determination were from Pharmacia. DEAE-cellulose DE-52 was from Whatman. Sodium dodecyl sulfate (SDS), acrylamide, methylenebisacrylamide and ammonium persulfate were from BDH Chemicals. *N,N,N',N'*-Tetramethylenediamine was from Kodak. *p*—Nitrophenyl-*p*-guanidobenzoate hydrochloride, Coomassie brilliant blue R 250, amidoblack solution and $(NH_4)_2SO_4$ were from Merck. Freunds complete adjuvant and *Mycobacterium tuberculosis* H 37 RA were from Difco Laboratories. All other chemicals were of the highest commercially available grade.

Analytical methods

Electrophoretic techniques. The electrophoretic assays were carried out in an LKB multiphore 2117 apparatus kept at a constant temperature of 5°C. For agarose gel and immunoelectrophoresis 1% gel plates, 8.4 × 9.4 cm, were prepared in Tris-barbiturate buffer of pH 8.6 (4-times concentrated stock solution: 22.4 g diethylbarbituric acid/44.3 g Tris/0.533 g calcium lactate/0.650 g sodium azide were made up to 1 l.) 10 V cm^{-1} were applied for 1 h. Fibrinogen-agarose gel electrophoresis for the specific detection of inhibitors has previously been described in detail [8,10]. In short, bovine fibrinogen was incorporated into a 1% agarose gel buffered with the described Tris-barbiturate buffer at final concentration of 0.675 mg fibrinogen per ml. After solidification of the agarose the gel plates were incubated at 80°C for 1 h in order to precipitate the fibrinogen. After electrophoretic resolution the plates were incubated in a solution containing the enzyme of choice at its appropriate concentration for 15 min (13 μg trypsin/ml; 20 μg chymotrypsin/ml; 26 μg elastase/ml; 0.2 U plasmin/ml) and then rinsed with distilled water for several hours. Undigested fibrinogen, which remained where protease inhibitors were located, was stained with amidoblack.

Analytical isoelectric focusing was made in polyacrylamide gel (LKB ampholine plate, pH 3.5—9.5) for 2 h at 25 W constant power, Anode solution: 1 M H_3PO_4. Cathode solution: 1 M NaOH. Gel was stained with 1% (w/v) Coomassie blue, dissolved into destaining solution (AcOH 8%/EtOH 25%/H_2O 67%).

Electrophoresis in SDS for molecular weight determination was carried out in 5% polyacrylamide gel buffered with 0.2 M sodium phosphate at pH 7.1. The molecular weight was calculated using the proteins of the Pharmacia calibration kit as reference substance. The gels were stained with 0.25% Coomassie brilliant blue R250 in 10% acetic acid and 45% methyl alcohol and destained in the same solvent mixture.

Molecular weight determination by gel filtration. A 93 × 2.5 cm column was packed with Sephadex G-100 and equilibrated with 0.1 M sodium phosphate of pH 7.4. It was calibrated with the Pharmacia calibration kit. The molecular weight was calculated according to Whitaker [11].

Assay for trypsin inhibitory capacity. Trypsin inhibitory capacity was measured according to a standard procedure [12] with *N*-benzoyl-arginine-*p*-nitroanilide as substrate. The volume of the assay was 3.0 ml. The concentration of trypsin was determined by measuring the absorbance at 278 nm. The molar extinction coefficient was taken as 33 680 [13] and the molecular weight as 23 900 [14]. The fraction of active enzyme was elucidated by titration with *p*-nitrophenyl-*p*-guanidobenzoate hydrochloride [15].

Protein concentration. Protein content was determined with the Folin reagent [16] with bovine serum albumin as a standard.

Immunochemical procedures. Antiserum against plasma proteins of the horse was obtained by immunization of a New Zealand white rabbit. 1 ml horse plasma was emulsified in 1 ml Freunds adjuvant to which 2 mg of killed *M. tuberculosis* were added. 3 weeks after the initial injection into the footpads the animal was boosted subcutaneously with the same antigen dose. 5 weeks after the initial injection the rabbit was bled.

Double gel diffusion and immunoelectrophoresis were done according to standard procedure [17].

Fractionation procedures. All steps were done in the cold room at temperatures between 4 and 8° C, with the exception of $(NH_4)_2SO_4$ precipitation, which was executed at room temperature.

Precipitation with $(NH_4)_2SO_4$. 250 ml saturated $(NH_4)_2SO_4$ solution were added to an equal volume of horse plasma. 6 h later the precipitate was separated by centrifugation at 50 000 × *g* for 45 min. The precipitate was discarded and the supernatant brought to 80% saturation by the addition of solid $(NH_4)_2SO_4$. After standing overnight the solution with the formed precipitate was again centrifuged. The second supernatant was discarded, the precipitate was dissolved in 25 ml water and dialyzed for 2 days, first against several changes of distilled water and then against 0.05 M Tris-HCl, pH 8.8, containing 0.05 M NaCl.

Chromatography on DE-52 cellulose. A 1.5 × 55 cm column was packed with DE-52 cellulose equilibrated with 0.05 M Tris-HCl, pH 8.8, and 0.05 M NaCl. The dialyzed product of $(NH_4)_2SO_4$ precipitation was applied and the column was first washed with 100 ml buffer. A linear gradient from 0.05 to 0.2 M NaCl was then washed through at a flow rate of 25 ml/h. Fractions were pooled as indicated in Fig. 2 and concentrated by ultrafiltration with a Millipore membrane to a volume of 5 ml and then dialyzed overnight against 0.05 M sodium acetate buffer, pH 6.0, containing 0.25 M NaCl.

Affinity chromatography on Sepharose-hemoglobin. By this procedure haptoglobin and cathepsin D were removed [18—20]. Horse hemoglobin was cross-linked to Sepharose 4B according to the method of March et al. [21]. A ratio of about 20 mg hemoglobin/ml cross-linked gel was attained. A column of 1.5 × 12 cm was packed and equilibrated with 0.05 M sodium acetate buffer of pH 6.0, containing 0.25 M NaCl. The applied sample consisted of 5 ml pooled and dialyzed material after passage through DE-52 cellulose. The resulting eluate was concentrated to 5 ml and dialyzed against 0.05 M sodium acetate buffer of pH 6.0, containing 0.25 M NaCl and 1 mM each of $MgCl_2$, $MnCl_2$ and $CaCl_2$.

Affinity chromatography on Sepharose-concanavalin A. A column of 1.5 × 12 cm was packed with con A-Sepharose and equilibrated with 0.05 M sodium acetate buffer of pH 6.0 containing 0.25 M NaCl and 1 mM each of $MgCl_2$, $MnCl_2$ and $CaCl_2$. 5 ml of the product after Sepharose-hemoglobin chromatography, were applied to the column. It was then washed with buffer until the absorbance of the eluate at 280 nm fell below 0.03. The glycoproteins were then eluted with 0.1 M 1-0-methyl-α-D-glucopyranoside. The resulting eluate was concentrated to 3 ml and dialyzed first against distilled water and then against 0.1 M sodium phosphate buffer, pH 7.4.

Gel filtration through Sephadex G-100. The same column which has been already described for the molecular weight determination was used. 3 ml of the product after Sepharose-con A chromatography was applied. The fractions containing inhibitory activity were pooled, concentrated, dialyzed against 0.05 M Tris buffer, pH 8.8, containing 0.005 NaCl and stored at —20°C.

Results

Fractionation of plasma

Salting out with $(NH_4)_2SO_4$ resulted in the precipitation of α-1 and α-2

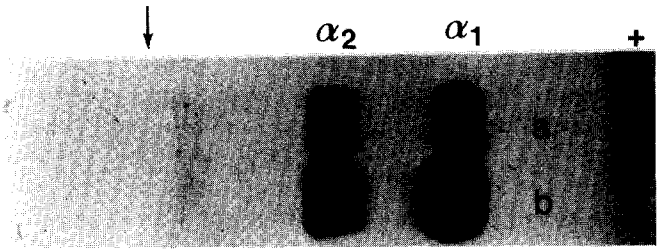

Fig. 1. Fibrinogen plate electrophoresis showing trypsin inhibition (a) by whole plasma and (b) by the fraction precipitated between 50 and 80% $(NH_4)_2SO_4$ saturation. Arrow: sample application.

inhibitors between 50 and 80% saturation (Fig. 1). Although α-1 and α-2 inhibitors could not be separated from each other by this step, the product was freed of slower moving material. Fractionation of the inhibitors was achieved by chromatography on DE-52 cellulose. The elution diagram is shown in Fig. 2. Little inhibitory activity was eluted before the application of the salt gradient which migrated in α-2 position. With the salt gradient three peaks of inhibitory activity were eluted. The electrophoretic mobilities of the inhibitors in selected fractions are shown in Fig. 3. α-2-inhibitor bands of different electrophoretic mobilities were eluted first. Then an α-1 band appeared in fraction 60 with two α-2 inhibitors still present. In fraction 85 one α-1 and one α-2 band were visible. Two α-1 bands could be recognized in fraction 90 and the α-2 bands vanished with further increasing fraction number.

Pools a to e from the DE-52 cellulose chromatography eluate were concentrated and further processed as indicated under Materials and Methods, through affinity chromatography and gel filtration through Sephadex G-100. The analytical results for pool e containing both α-1 inhibitor bands are reported here. The α-2 inhibitors are still under investigation and the results will be published

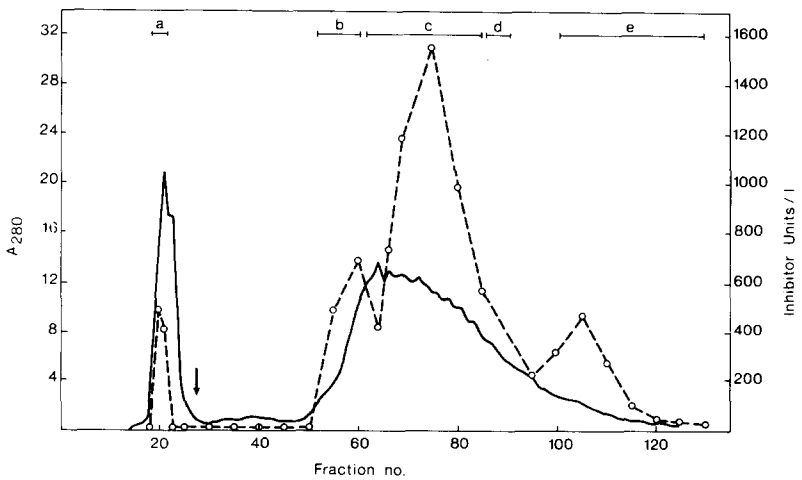

Fig. 2. DE-52 cellulose chromatography of the fraction precipitated between 50 and 80% $(NH_4)_2SO_4$ saturation. ———, absorbancy at 280 nm; - - - - - -, trypsin inhibition. Arrow: start of gradient. a, b, c, d, e; pools of fractions.

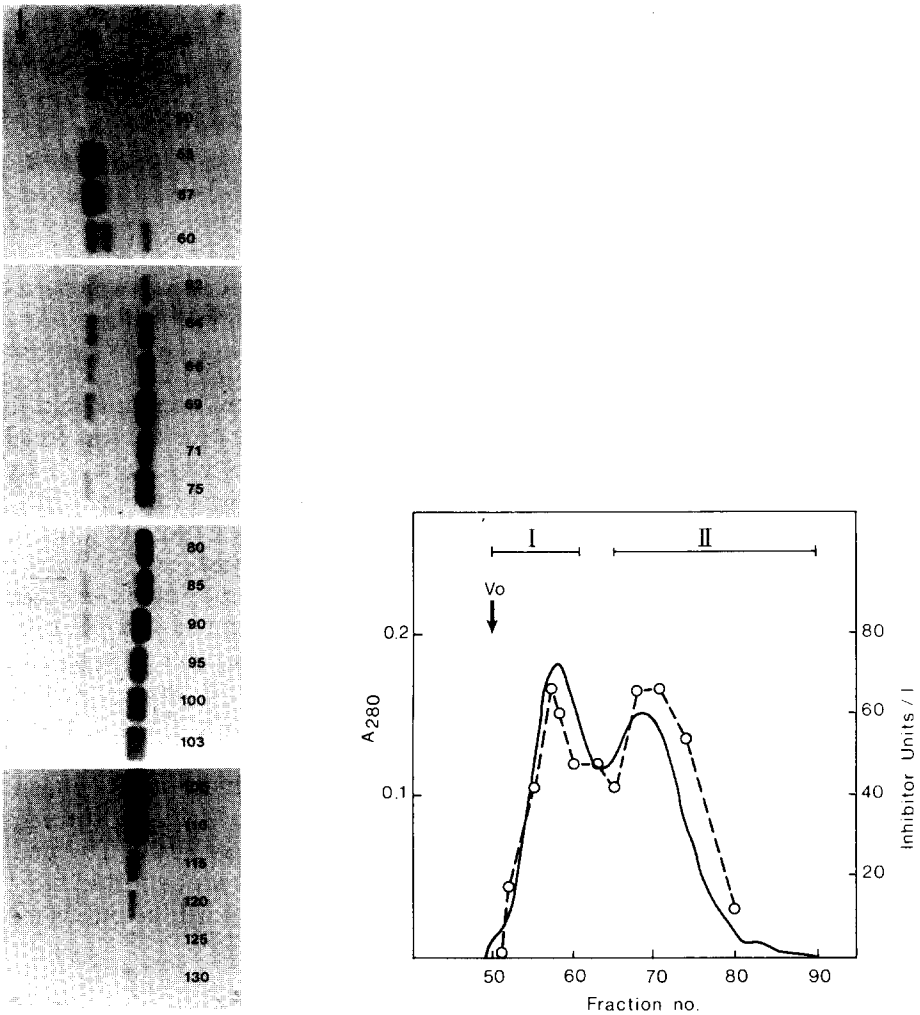

Fig. 3. Fibrinogen plate electrophoresis showing trypsin inhibition of selected fractions after DE-52 cellulose chromatography. The numbers correspond to the fraction numbers in Fig. 2. Arrow: sample application.

Fig. 4. Sephadex G-100 gel filtration of pool e after DE-52 cellulose chromatography, which was further purified by affinity chromatography (see text for details). ———, absorbance at 280 nm. - - - - - -, trypsin inhibition. V_0: exclusion volume. I: peak I. II: peak II.

later. The elution diagram of pool e after Sephadex G-100 gel filtration consisted of two peaks (I and II) in approx. equal amounts (Fig. 4). Peak I consisted of the slower migrating inhibitor band, whereas the faster migrating one was located, together with the residual contaminating activity of an α-2 inhibitor, in peak II (Fig. 5). Agarose gel electrophoresis stained for proteins confirmed the presence of two protein bands corresponding to both α-1 inhibitors and of a faint amount of protein in the α-2 position (Fig. 6). Immunoelectrophoresis confirmed these results (Fig. 7). With polyvalent antiserum to horse plasma proteins, a single precipitation arc in the α-1 position indicated homo-

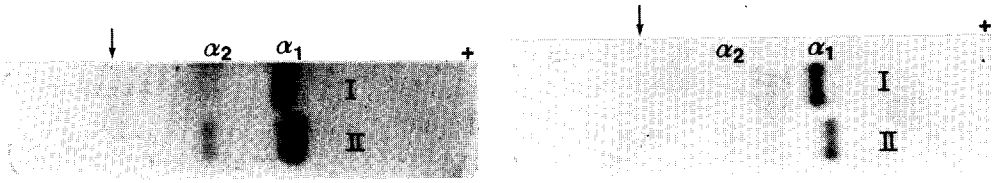

Fig. 5. Fibrinogen plate electrophoresis of peak I and of peak II eluted from Sephadex G-100. Arrow: sample application.

Fig. 6. Agarose gel electrophoresis showing protein fractions of peak I and of peak II eluted from Sephadex G-100. Arrow: sample application.

Fig. 7. Immunoelectrophoresis of peak I and of peak II eluted from Sephadex G-100 (wells). Antiserum (AS) against horse serum proteins (through).

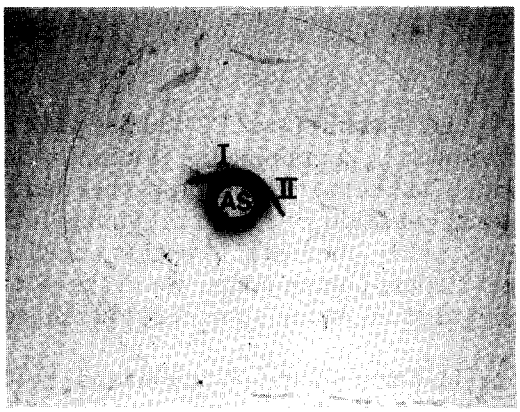

Fig. 8. Double gel diffusion of peak I and of peak II eluted from Sephadex G-100. Antiserum (AS) against horse serum proteins. The precipitation line of the contaminant present in peak II (see Fig. 7) was present, but is not clearly visible on the photograph.

geneity by this criterium of the α-1 inhibitor in peak I. Peak II gave two arcs of precipitation, corresponding to the α-1 and the α-2 inhibitory fractions.

An important question to be solved was the antigenic relationship of the slow and the fast α-1 inhibitors. Double gel diffusion revealed that the slow and the fast α-1 inhibitors had identical antigenic determinants when they were tested with the same polyvalent antiserum which was used for immunoelectrophoresis (Fig. 8).

Isoelectric focusing showed a banding pattern of a limited heterogeneity with 7 bands focused between pH 4.6 and 5.0 for peak I. 4 additional bands between pH 5.0 and 5.8 were present in peak II, demonstrating contaminating

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Fig. 9. Isoelectrofocusing pattern of peak I (left) and of peak II (right) eluted from Sephadex G-100.

material of higher isoelectric point (Fig. 9).

Estimation of the molecular weight by gel filtration was 90 000 for peak I, the slow moving inhibitor, and 65 000 for peak II, containing the fast inhibitor. The slow moving inhibitor of peak I, which had homogeneous properties determined by the previously described criteria, consisted of three protein bands in SDS-polyacrylamide gel electrophoresis with molecular weights ranging between 60 000 and 65 000. Since peak II was contaminated we declined to analyze it by SDS electrophoresis, feeling that no information could be gained thereby.

The enzyme specificities of both α-isoinhibitors were also compared. Both inhibitors abolished the activity of trypsin, chymotrypsin and elastase, estimated by fibrinogen plate electrophoresis. By the same method no inhibition of plasmin could be detected, confirming our previous results that the α-1-protease inhibitors of the horse inhibit plasmin very poorly [8].

Discussion

The method of fibrinogen-agarose gel electrophoresis [22,23] has been modified and adapted by us [8,10] to high sensitivity and resolving power. It has the great advantage of direct visualization of the inhibitory function of natural protease inhibitors with any desired protease. The application of this adapted method has to be regarded as a new approach, adding a new dimension to the investigation of protease inhibitors. Certain discrepancies with results obtained by other methods, which are currently used, may therefore not yet be harmonized.

Starting with the serum from a horse having a single α-1-protease inhibitor band [8] biochemical fractionation uncovered two isoinhibitors. The doubling of the band became unequivocally apparent with the elution from DE-52 cellulose chromatography. Yet both isoinhibitor bands were eluted together and could not be separated chromatographically. From Sephadex G-100 gelfiltration, however, they were differently eluted: the slow inhibitor band at a position corresponding to a molecular weight of 90 000, the fast band at the 65 000 position. By SDS electrophoresis the molecular weight of the slow inhibitor band was 60 000—65 000, which was in disaccord with the above mentioned value. However, the value coincided with the molecular weight of the fast inhibitor band. A molecular weight of 60 000 has also been reported by Ek [24] for the P_Y fraction * of horse blood, which has protease inhibiting capacity, migrates in agarose gel electrophoresis in the albumin region and is regarded as the analog of human α-1-antitrypsin. On the other hand the fast and the slow inhibitor were closely related, since they were immunochemically indistinguishable and had an identical inhibitor spectrum.

The above mentioned physical differences could be explained either by different conformations of the slow and the fast band, the fast band being more 'compact' than the slow band [25], or by dimer formation. A third possibility, although remote, could consist of partial adsorption of the retarded peak II to Sephadex G-100 in addition to molecular sieving.

The first assumption of different conformations gets some support through an observation with human α-macroglobulin [25]. A fast and a slow form of α-2-macroglobulin could be differentiated according to their electrophoretic migration. The slow form was active, whereas the fast form had no inhibitory activity. Treatment of the slow form with protease or with NH_4^+ changed it to the fast form. On prolonged storage a gradual change from the slow to the fast form occurred with the appearance of forms with intermediate electrophoretic mobilities. No dissociation of the molecule was observed and no change in the isoelectric point was registered, so that the difference in electrophoretic mobility was exclusively attributed to a conformational change of the tertiary structure: the fast form being more 'closed' or 'compact' than the slow form.

If the properties of the two forms of α-2-macroglobulin are compared with our results, there are similarities but also some differences. The similarity is the possibility that doubling of the α-1-protease inhibitor band is a consequence of fractionation, since in the original serum one band was observed. The difference is the fact that both bands of horse α-1-protease inhibitor had inhibitory activity.

The second supposition of dimer formation is challenged by the fact that a molecular weight of 90 000 of the presumptive dimer was measured instead of the expected 130 000. This, however, could be accounted for by assuming dimer formation coupled with conformational changes.

The link between the functional heterogeneities of horse α-1-protease inhibitors, as they previously have been revealed by us [8] and as they now are being described, with the extensive polymorphism of fast migrating protein bands of

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* The P_Y fraction is the designation for the P_i analog of man.

serum visualized by acidic starch gel electrophoresis [26], isoelectric focusing [27], or by antigen-antibody crossed electrophoresis [28] is very difficult to establish. Ek [24] could relate the P_r system of multiple prealbumin protein bands of the horse to antitrypsin activity, which was migrating as a broad band in the albumin region in agarose gel electrophoresis at pH 8.6. This leaves little doubt that the P_r system of the horse is analogous to the P_i system of the α-1-antitrypsin of man. Recently the horse P_r system has been further differentiated by two-dimensional electrophoresis, combining low pH in agarose and high pH in polyacrylamide gel [29]. By this method the P_r zone could be subdivided into P_i1 bands and P_i2 bands. A slower migrating portion of presumed P_i1 bands was assumed to consist of P_i1 dimers. The relation of these findings to our present results however remains obscure.

Finally, some unusual features of α-2-protease inhibitors have to be mentioned, which were revealed as a 'byproduct' of fractionation. In the first step of $(NH_4)_2SO_4$ fractionation an important part of α-2-protease inhibitors was precipitated at the same fractional saturation (50—80%) as the α-1-protease inhibitors, although it has been reported that in the horse α-2-macroglobulin is removed at a lower saturation between 33 and 45% [30]. On DE-52 cellulose chromatography three α-2 inhibitor bands with slightly different electrophoretic mobilities were eluted. At least one of these did not have an elevated molecular weight, since later on it was eluted as a contaminant of the α-1-protease inhibitor at the 65 000 position in the Sephadex G-100 eluate. The properties of the α-2-protease inhibitors of horse plasma are now being further investigated in this laboratory.

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