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IDENTIFICATION OF THE PR PREALBUMIN PROTEINS IN HORSE SERUM

By

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EK, NILS: *Identification of the Pr prealbumin proteins in horse serum.* Acta vet. scand. 1977, *18*, 458—470. — The Pr protein, which is one of the major equine acidic prealbumins and which consists of a large number of phenotypes, has been studied with regard to its chemical identity.

Serum samples of known Pr phenotype which had been treated with varying amounts of bovine trypsin were subjected to starch gel electrophoresis at pH 4.8. When a certain amount of trypsin was used, the Pr protein was markedly affected, whereas the other acidic prealbumins retained their normal electrophoretic pattern.

Extracts from three different regions of the acidic prealbumin field were tested by the casein precipitating inhibition test (CPI-test). Marked antitrypsin effect appeared against the extract from the Pr zone but not against the extracts from the two other acidic prealbumin zones.

When acidic starch gel electrophoresis was combined with the CPI-test, a broad inhibitory zone appeared in the area of the Pr proteins.

Pr protein was isolated by the use of agarose gel electrophoresis and sepharose column chromatography. The isolated protein which was tested for purity by gel electrophoresis had a molecular weight of about 60,000.

It is concluded that the equine Pr protein corresponds to α_1-antitrypsin.

horse; Pr prealbumin; antitrypsin activity.

The designation prealbumin was originally adopted by *Smithies* (1955) for those zones of proteins which migrate ahead of albumin in alkaline gels. *Brænd & Efremov* (1965) and *Gahne* (1966) working with acidic starch gels, detected a large number of horse serum protein zones migrating faster than the albumins. Of these the fastest migrating ones belong to one genetic system called the Pr system (*Gahne, Brænd* 1970).

Fagerhol & Brænd (1965) also using acidic starch gels, described a genetic system of prealbumins in human serum. This protein is identical with α_1-antitrypsin and the term Pi (protease inhibitor) has been chosen for the corresponding genetic system (*Fagerhol & Brænd* 1966).

Preliminary studies indicated that the Pr proteins in horse serum correspond to α_1-antitrypsin (*Brænd* 1967).

The present report deals with investigations aimed at identification of the equine Pr proteins.

MATERIALS AND METHODS

Sera

Blood samples, obtained from healthy horses, were allowed to coagulate and the sera collected after centrifugation. The serum samples were stored at —20°C when not in use. Serum samples from a Døle horse of Pr phenotype FS (*Brænd* 1970) were used for the prealbumin zone protein preparations and investigations.

Starch gel electrophoresis of trypsin treated samples

Trypsin from bovine pancreas (type III, 2 × crystallized, lot 97 B - 8000, Sigma Chemical Company, St. Louis, Mo., USA) was diluted in phosphate buffer 0.01 M, 0.15 M-NaCl, pH 7.5 to an amount of 125 mg per ml and then in four 2-fold serial dilutions. Each of the trypsin solutions was mixed with a sample of horse serum of Pr phenotype FS (1.8 ml) in proportion 1:10 and incubated for 30 min. at 37°C. The samples were run in parallel on starch gels with a control sample without any trypsin. Technique and nomenclature were as described by *Brænd* (1970).

The agar-casein-precipitating method (Sandvik 1962*)*

A hot solution containing 1.40 % agar (Bacto-Agar, Difco), 1 % sodium caseinate (Eastman Kodak P 914) (prepared initially as a 4 % solution and adjusted to pH 6.5 with HCl), 0.01 % merthiolate, and 0.003 M-$MgCl_2$ was poured into a glass tray to a depth of 2 mm and allowed to solidify. Circular wells of 6 mm diameter were cut in the agar layer with a cork borer, for determination of proteolytic enzyme activity. The enzyme preparations were placed in the wells with a micropipette.

Preparation of acidic prealbumin fractions

A 1 ml sample of horse serum was absorbed into a 13×0.5 cm strip of filter paper of about 1 mm in thickness and subjected to electrophoresis on starch gel (*Brænd* 1970). Immediately after the run, three strips each about 1 cm broad, were cut out of the gel at right angles to the direction of run. One was taken from the Pr zone, the second from the middle of the total acidic prealbumin region (Xh) and the third from the slowest migrating protein zone, designated Xk (*Brænd* 1970). A gel previously run in the same way and stained with amido black was used as a marker for the position of the zones.

The strips were cut into pieces of about 1×1 mm, suspended in 10 ml phosphate buffer 0.05 M, pH 7.4 and stored at — 20°C. The samples were thawed and centrifuged at 5000 r. p. m. for 15 min. in special plastic tubes equipped with a filter disk. The clear solution from each sample was concentrated to a final volume of 1 ml by applying Aquaside (Calbiochem) as a dehydrating agent around a dialyzer tubing with the solution inside.

Detection of inhibitory proteolytic activity

Trypsin (0.025 ml corresponding to 0.25 mg per ml) was placed in a middle well in the caseinate agar and 0.025 ml of the specially prepared serum fractions in adjacent wells. The plate was then covered with a tightly fitting glass lid and incubated at 37°C for 18 hrs. Proteolytic enzyme activity is indicated by a distinct zone of precipitation around the middle well in the otherwise completely transparent agar, while inhibition is indicated by a more or less narrowing of the white precipitation zone.

Numerical expression of the inhibitory enzyme activity

This was obtained by using the casein precipitating inhibition test (CPI-test) as described by *Fossum* (1970 a). The CPI-test was performed by placing filter paper strips moistened with different dilutions of serum and serum fractions on the agar surface, and afterwards placing strips with enzyme solutions at right angles to the inhibitors. A semi-quantitative measure for the inhibitory capacity was obtained by determination of the highest dilution of whole serum and of the serum fractions resulting in inhibition.

Electrophoretic CPI-test

A modification of Fossum's method using starch gel electrophoresis instead of paper electrophoresis was employed. A horse serum sample Pr phenotype FS was subjected to electrophoresis on starch gel pH 4.8 as previously described (*Brænd* 1970). One 1.5 cm broad strip containing the total acidic prealbumin region, was cut from the gel along the running direction and immediately transferred to the surface of the caseinate medium. After incubation at 37°C for 2—3 hrs. the gel strip was removed from the medium and replaced by a narrow (3 mm) strip of filter paper which was moistened with trypsin solution (1 mg per ml). The caseinate medium was allowed to stand at 37°C for 18 hrs. Precipitation zones occurred along the enzyme containing strip. Inhibition is indicated by a narrowing of the precipitation zones.

Isolation of the Pr fraction

A sample of horse serum Pr phenotype FS (30 ml) was run in electrophoresis in agarose gel. The serum sample was mixed with starch and applied in a 2 cm broad trough in an agarose gel $30 \times 40 \times 1$ cm. The sample was run for 72 hrs. with conventional barbital buffer (pH 8.6) and at a voltage of 250 v. A sample of 1.0 ml serum was mixed with 0.1 % bromophenolblue (2:1) and run in parallel as a marker of the albumin.

After the run, strips each 1 cm broad were cut out of the gel at right angles to the direction of migration. A zero line was drawn in the middle of the albumin zone.

The strips were treated as previously described for the extracts prepared from starch gels after electrophoresis. Each sample was concentrated to a volume of 5 ml and tested for antitrypsin activity by the method earlier described.

The fraction with the highest activity was pooled and albumin removed by affinity chromatography on a column of sepharose 4B — blue dextran conjugate according to the method of *Travis & Pannell* (1973). The sample was applied to a column (1.5×30 cm) equilibrated with 0.05 M Tris-HCl, 0.15 M-NaCl, pH 8.0. The eluate was concentrated to a final volume of 2 ml.

Tests of the Pr preparation

The isolated Pr preparation was run in parallel with horse serum on an acid starch gel by the method earlier mentioned.

In addition, the preparation was run in agarose gel electrophoresis (20 v per cm) in 1 mm thick layers of gel on water cooled glass plates with conventional barbital buffer (pH 8.6), and was examined by immunoelectrophoresis against anti-horse serum by a method previously reported (*Ek* 1974).

The protein fraction (0.5 ml) was applied to a column (0.9 × 60 cm) of Sephadex G-100 with 0.01 M acetate buffer, pH 6.0. The molecular weight was determined using the method described by *Whitaker* (1963). The reference proteins were bovine albumin (molecular weight (mol. wt.) 70 000), ovalbumin (mol. wt. 45 000), pepsin (mol. wt. 35 500) and α-chymotrypsin (mol. wt. 22 500).

The nitrogen content was determined by the Micro-Kjeldal method (*Hawk* 1965) and the optical density calculated at 280 nm using the Beckman spectrophotometer DB.

The inhibitory enzyme activity in the Pr preparation and in whole serum against three different proteolytic enzymes, bovine trypsin (0.050 mg per ml), α-chymotrypsin from bovine pancreas (0.005 mg per ml, type I, 3 × crystallized, Sigma Chemical Com-

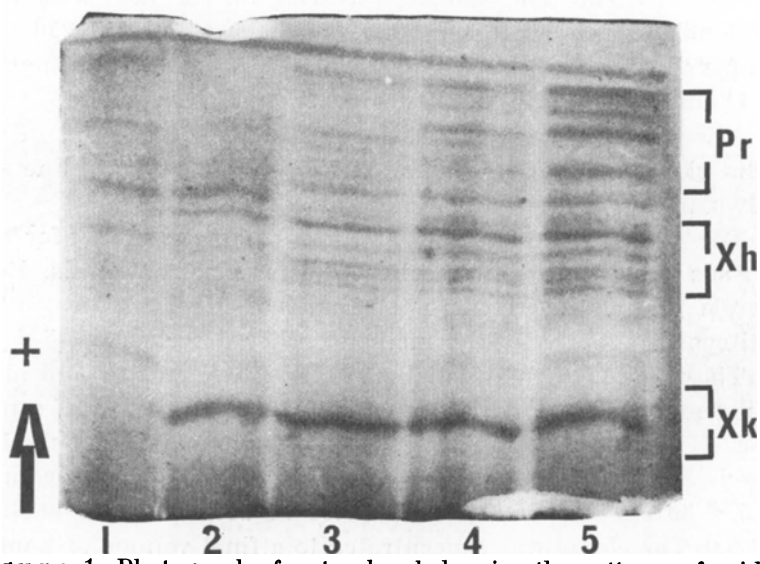

Figure 1. Photograph of a starch gel showing the patterns of acidic prealbumins after electrophoresis of trypsin treated samples. Bovine trypsin additions were 125 mg per ml, ratio 1:10 for sample 1, then 2-fold serial dilutions for samples 2—4. Sample 5 is a control without any trypsin.

pany*) and Bacillus subtilis proteinase (0.001 mg per ml, type VIII, crystallized, Sigma Chemical Company*) was determined by means of the CPI-test as earlier described (*Fossum* 1970 a).

Photographic techniques

Photographs were taken on Polaroid films type 107 by dark-field illumination.

RESULTS

Fig. 1 is a photograph of a stained starch gel on which trypsin treated horse serum samples have been run. The photograph shows a comparison between the normal pattern of a Pr FS phenotype and the patterns of the same phenotype after it had been treated with increasing amounts of trypsin. In the sample with the highest concentration, all the original zones have either disappeared or changed positions, resulting in a completely new pattern. The effect apparently concerns all the acidic prealbumins, including those of the Xk system. With the lowest concentrations of trypsin the Pr proteins are the first ones to be affected, whereas the Xk proteins and other acidic prealbumin zones appear with the normal patterns.

When trypsin and extracts of the Pr, the Xh and Xk starch gel regions respectively were placed in adjacent wells in casein

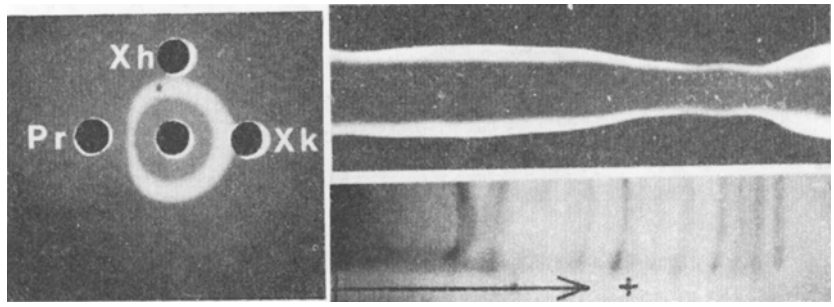

Figure 2. Left: Demonstration of trypsin inhibitory effect in extract from the prealbumins of horse serum. Middle well: Bovine trypsin (0.25 mg per ml).
Right: Electrophoretic casein precipitating inhibitory test of prealbumins with acidic starch gel (pH 4.8). The enzyme used is bovine trypsin (1 mg per ml). Below a strip of the gel stained with amido-black.

* Sigma Chemical Company, St. Louis, Mo., USA.

agar, a flattening of the precipitation zone around the trypsin well only occurred against the extract of the Pr region (Fig. 2). This indicates an antitrypsin effect by the proteins of this system and little or no inhibition activity of the proteins of the two other prealbumin zones.

Fig. 2 also shows the electrophoretic pattern of the inhibitors against bovine trypsin in the prealbumin zone. There is a clear inhibition against the Pr zone, but the inhibition is extended into the area behind the known Pr bands. A weaker zone of inhibition can be seen near the line of application.

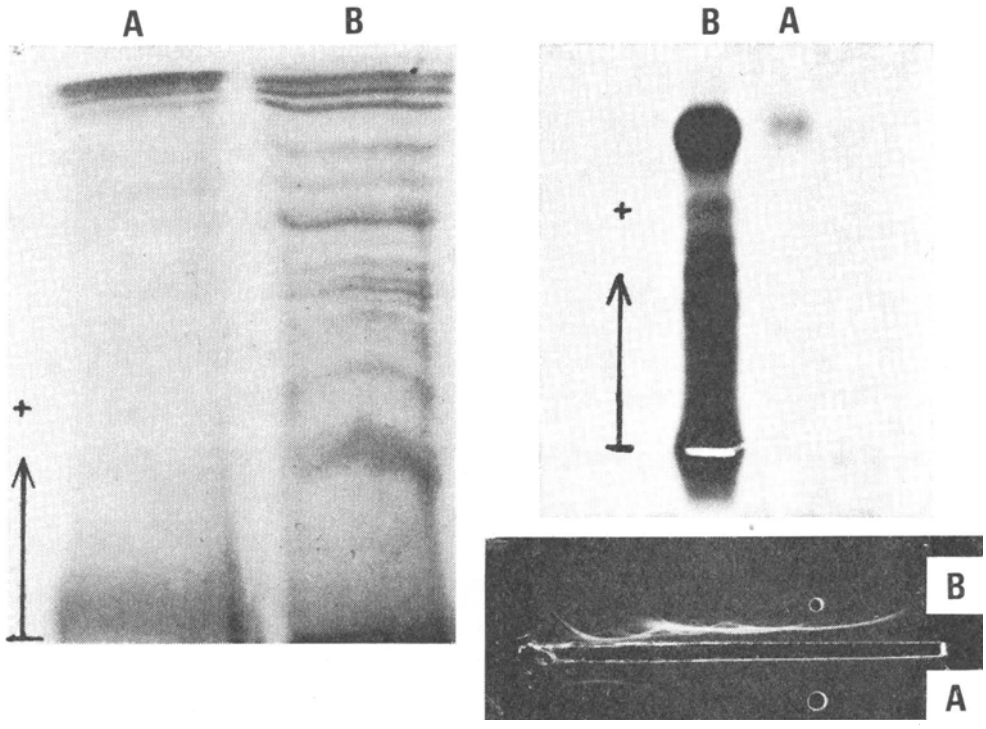

Figure 3. Electrophoretic tests of the isolated Pr protein fraction in parallel with whole horse serum.
A: The Pr protein fraction
B: Whole horse serum
Left: Photograph of a gel showing electrophoresis on acid starch gel (pH 4.8.).
Right top: Photograph taken after electrophoresis on agarose gel with barbital buffer (pH 8.6).
Right bottom: Photograph showing immunoelectrophoresis on agarose gel with barbital buffer (pH 8.6) against anti-horse serum.

When the isolated Pr preparation was run on starch gel, three protein bands appeared in the front of the field (Fig. 3). Their location corresponds to the three F bands in the Pr zone. It can further be seen that the first one is stronger and broader than the others, whereas in the serum sample they appear approximately equal.

On the original gel, two weak bands could be seen behind the three in the front, but they are not visible on the photograph.

In electrophoresis on agarose gel with barbital buffer (pH 8.6), the isolated protein fraction appeared as a broad band which migrated at the same speed as the front of the albumin zone. Finally the isolated protein gave only a single precipitin line after immunoelectrophoresis with anti-horse serum (Fig. 3).

When the isolated protein fraction was applied to gel filtration on a column of Sephadex G-100 only one peak appeared (Fig. 4). The molecular weight was calculated to be about 60 000, which is at the same level as reported for α_1-antitrypsin in human serum (*Travis et al.* 1974).

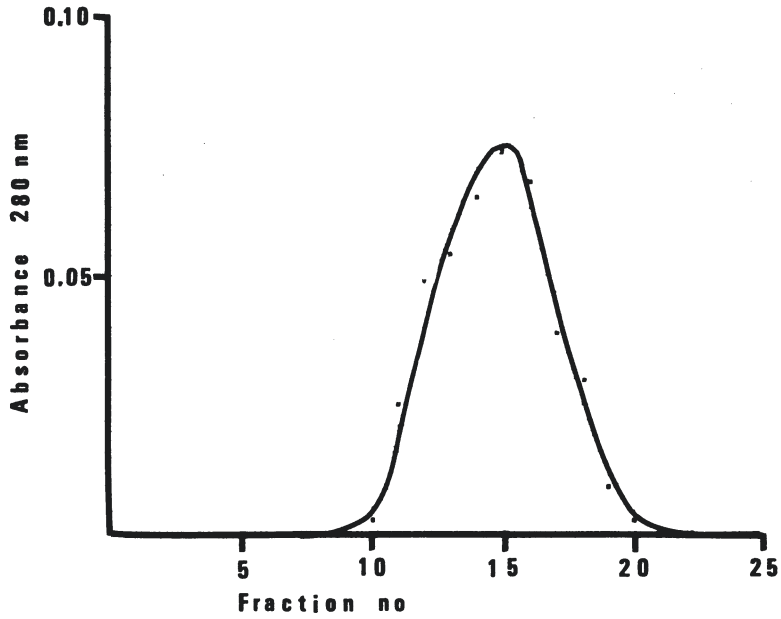

Figure 4. Gel filtration of the Pr protein on Sephadex G-100 on a column (0.9 × 60 cm) with acetate buffer 0.01 M, 0.1 M-NaCl, pH 6.0. Fractions of 1 ml were collected.

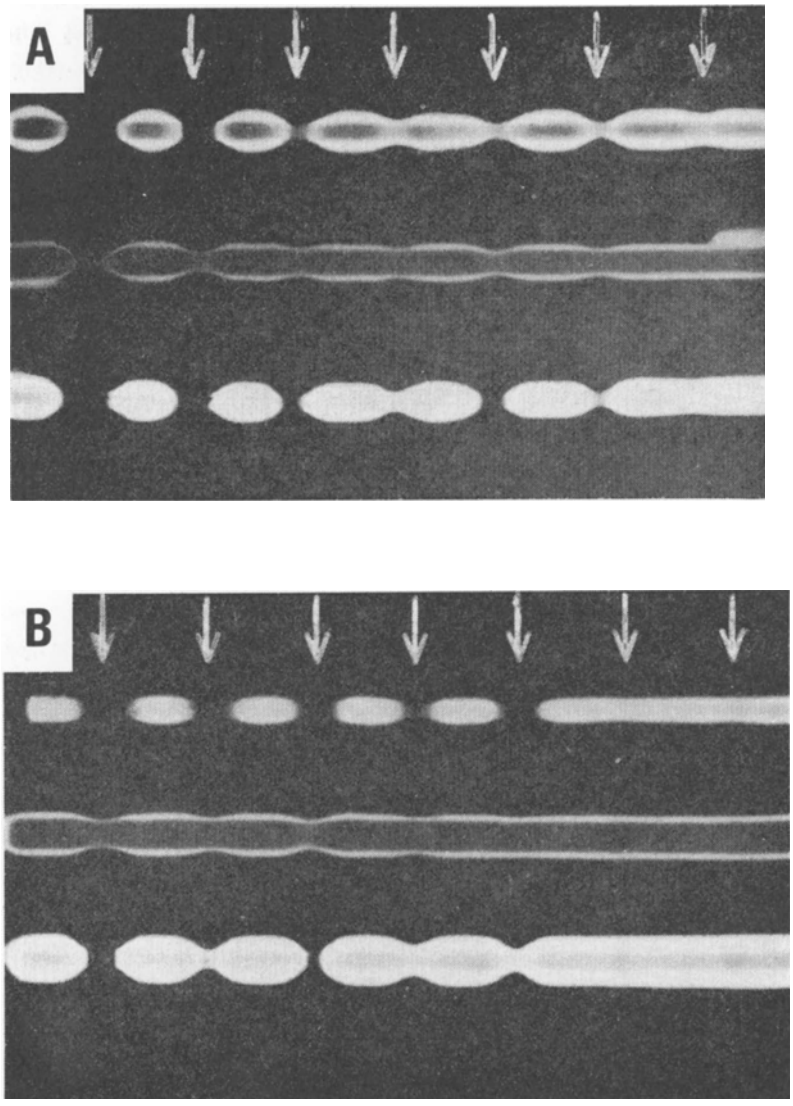

Figure 5. Photographs showing crosswise CPI-test with different dilutions of whole horse serum (A) and the isolated Pr protein fraction (B).

The dilutions are (from left to right as marked by arrows) 1:1, 1:10, 1:50, 1:100, 1:200, 1:500 and 1:1000.

Enzymes are downwards: Bovine trypsin (0.05 mg per ml), α-chymotrypsin (0.01 mg per ml) and proteinases from Bacillus subtilis (0.001 mg per ml). Enzymes are the same in A and B.

The protein concentration of the sample was determined as 1.5 mg per ml and the optical density at 280 nm as 1.04. Thus the optical density of a 1 % concentration of the Pr fraction at 280 nm could be calculated as 6.93.

When testing the protease inhibitory activity it was found that the highest dilution of the protein fraction resulting in inhibition against bovine trypsin was 1:200, against α-chymotrypsin 1:100 and against Bacillus subtilis protease 1:200, whereas the corresponding dilution figures for whole horse serum were trypsin 1:500, α-chymotrypsin 1:200 and Bacillus subtilis protease 1:500 (Fig. 5).

DISCUSSION

The reason why a horse serum of Pr phenotype FS was chosen for these studies was that the F bands are the fastest ones with the acidic starch gel electrophoretic technique. It was therefore assumed that the zones to be isolated would contain less of other proteins than more slowly migrating Pr phenotypes.

One of the aspects of the present studies was to investigate whether the Pr protein might possess antitrypsin effect. The purpose of adding trypsin to horse serum was to observe the influence on the protein bands in the prealbumin field. When complexes between trypsin and proteins with antitrypsin effect are formed, changes of the electrophoretic pattern would be expected.

When bovine trypsin was added to horse serum, interaction first took place for the proteins of the Pr system, since the original Pr bands in this area disappeared while new bands of a slower mobility came into view. These new zones might have been complexes between the inhibitor and trypsin forming new components with different mobilities. *Travis et al.* (1974) when adding increasing quantities of human trypsin to human α_1-antitrypsin found by gel slab electrophoresis a new component with intermediate mobility. This component would break down in the presence of excess trypsin. Similarly, in the present studies the more or less total disappearance of the original prealbumin zones in the presence of excess trypsin might have been due to trypsin digestion of the original proteins.

The inhibition against bovine trypsin when determined after acidic starch gel electrophoresis seems to be located in a rather

broad zone extending from the known Pr zone in a cathodal direction (Fig. 2). *Fagerhol* (1969) found by antigen-antibody crossed electrophoresis that in human serum the Pi allele product of the α_1-antitrypsin system was a pattern of eight zones. If similar multiband phenotypes exist in horses, more zones hitherto not recognized may belong to the Pr fraction. This could then explain the extent of the inhibitory area.

The weak inhibitory activity near the start line (Fig. 2) may be due to other protease inhibitors in serum. *Fossum* (1970 b) investigating sera of different species by paper electrophoresis with phosphate buffer pH 6.2, found two trypsin inhibitors. As well as a strong rapidly migrating inhibitor, a weak slowly moving inhibitor appeared which was thought to be identical with the α_2-trypsin inhibitor.

The pattern of the isolated protein run on acid starch gel was not quite identical with that of the Pr fraction in whole horse serum (Fig. 3). A possible explanation is that some changes in the molecules took place during the isolation procedure, influencing their electrophoretic properties. However, taking into consideration the additional weak bands visible on the original gel, the pattern of the isolated protein corresponded in its broad features to the Pr protein of phenotype FS in whole horse serum.

In electrophoresis on agarose gel with alkaline buffer the protein migrated in front of albumin. Using a similar electrophoretic technique on human serum the Pi protein is located behind albumin in the α_1-zone (*Fagerhol & Laurell* 1967). This indicates a difference between horse and human serum as regards migration rates of α_1-antitrypsin and albumin.

The inhibition against trypsin in the protein fraction was found to be about the half of the activity of whole horse serum (Fig. 5). In human serum α_1-antitrypsin has been found to account for at least 70 % of the total inhibitory activity (*Travis et al.* 1974). In normal human serum a concentration of 1.80 mg per ml α_1-antitrypsin has been reported (*Anon.* 1966). Considering the protein concentration found in the isolated protein fraction (1.5 mg per ml), there seems to be accordance between the data obtained from the isolated Pr protein in the present studies and the α_1-antitrypsin in human serum.

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SAMMENDRAG

Identifisering av Pr prealbumin proteiner i hesteserum.

Pr-proteinet er et av de fremtredende proteiner i gruppen sure prealbuminer i hesteserum og har et stort antall fenotyper. Proteinet er undersøkt med henblikk på dets kjemiske identitet.

Serumprøver av en kjent Pr-fenotype som var blitt behandlet med varierende mengder med bovint trypsin, ble kjørt på stivelsesgel-elektroforese ved pH 4.8. Ved tilsetning av en bestemt mengde trypsin, ble det en tydelig påvirkning på Pr-proteinene, mens de øvrige sure prealbuminer beholdt sitt normale elektroforesemønster.

Ekstrakter fra tre forskjellige soner av feltet med de sure prealbuminer ble testet ved hjelp av casein precipitation inhibition test (CPI-test). En tydelig antitrypsinvirkning viste seg mot ekstraktet fra Pr-sonen, derimot var det ingen reaksjon mot ekstraktene fra de to øvrige prealbuminsoner.

Ved bruk av elektroforese på sur stivelsesgel kombinert med CPI-test fremkom en bred hemningssone i Pr-proteinenes område.

Pr-protein ble isolert ved hjelp av elektroforese på agarosegel og sepharose-kromatografi. Renheten av det isolerte protein som hadde en molekylvekt på omkring 60 000, ble testet ved hjelp av gelelektroforese.

Det konkluderes med at Pr-proteinet i hesteserum tilsvarer α_1-antitrypsin.

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