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The Equine Protease Inhibitory System (Pi): Abnormal Expressions of Pi^F, Pi^L, and Pi^{S_1}

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Three cases of abnormal expression of the equine protease inhibitory alleles, Pi F, L, and S_1, were observed following the examination of 30,000 plasma samples by one-dimensional acid (pH 4.6) polyacrylamide gel electrophoresis. Characterization of the abnormal proteins in terms of isoelectric point, molecular mass, inhibitory spectra, and sialic acid content was performed using one- and two-dimensional electrophoretic techniques. The Pi F and S_1 abnormalities were postulated to be the result of amino acid substitutions causing alterations in the processing of the carbohydrate side chains. No explanation could be offered for the Pi L abnormality other than a charge shift mutation. Abnormal types, F, L*, and S_1^* behaved as alleles but the distribution of L* in offspring from one stallion (present in only 6 of 83 offspring) differed significantly from expectation.*

KEY WORDS: equine; α_1-protease inhibitor; abnormal expression; mutation; altered carbohydrate processing.

INTRODUCTION

The major proteins of the equine protease inhibitory system (Pi) have been characterized by ISO-DALT electrophoresis in terms of isoelectric point (pI), molecular mass (M_r), and inhibitory spectra (Pollitt and Bell, 1983a, b). Eight alleles, designated Pi F, G, I, L, N, S_1, S_2, and U, have been described in the Thoroughbred and an additional 10 alleles have been found in the Stan-

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dardbred (Bell *et al.*, 1984). Juneja *et al.* (1979) postulated on the basis of differences in M_r and inhibitory spectra that the equine Pi proteins were controlled by two closely linked loci.

Extensive studies by Pollitt and Bell (1983a, b) and Bell *et al.*(1984) revealed exceptions to the two loci theory based on inhibitory spectra. Three classes of equine Pi proteins were described—class I inhibiting trypsin, chymotrypsin, and elastase; class II inhibiting trypsin and elastase; and class III inhibiting only chymotrypsin (Pollitt and Bell, 1983b). Of the eight "Thoroughbred" alleles, four (G, L, S_1 and S_2) do not code for class II proteins, whereas all eight code for class I and class III proteins.

Apparent abnormal expressions of three Pi alleles, F, L, and S_1, were found following the examination of 30,000 equine plasma samples. The abnormalities were studied by isoelectric focusing (IEF) (*p*H 3.5–6.0 and 4.2–4.9), alkaline and acid one-dimensional (1D) and two-dimensional (2D) polyacrylamide gel electrophoresis, and treatment of normal and abnormal plasmas with neuraminidase. The detection of the abnormalities afforded us the opportunity to investigate the relationships of the various Pi components, to gain insight into their genetic control, and to study the microheterogeneity of the Pi proteins. Preliminary reports of the abnormal expressions have been presented (Patterson and Bell, 1984, 1985).

MATERIALS AND METHODS

Plasmas

Equine blood samples, which were air freighted to our laboratory for the purpose of identification and parent verification by blood typing, were the source of plasmas. The plasmas were removed as soon as practicable following receipt of the samples and stored frozen at −20°C until examined electrophoretically. Their protease inhibitor (Pi) types were determined by acid polyacrylamide gel electrophoresis as described by Bell *et al.* (1984). After Pi typing, the cases of abnormal expression were further analyzed by the techniques of isoelectric focusing and horizontal polyacrylamide gel electrophoresis.

Isoelectric Focusing

Isoelectric focusing (IEF) was carried out in a flat-bed focusing apparatus (LKB Ultrophor, Model 2217) using a power supply capable of delivering 2500 V, 250 mA, and 110 W (LKB Model 2197) with a refrigerated cooling unit (Julabo Model F20H) to maintain the cooling plate at constant temperature. The coolant temperature was set at 10°C but was increased to 23°C for the final 30 min of isoelectric-point determinations.

Ultrathin IEF gels (0.25 mm) in the *p*H range 3.5–6.0 (LKB), which were the first dimensions of subsequent two-dimensional analyses, were prepared and run according to the method described by Pollitt and Bell (1983a) except that focusing was usually allowed to proceed for a longer period of 210 min. For *p*I determinations, thicker gels (0.7 mm) polymerized with ammonium persulfate and TEMED were used. IEF gels of 0.25-mm thickness were also run in the *p*H range 4.2–4.9 (Pharmacia) and prepared according to the manufacturer's directions.

Isoelectric-Point Determinations

Calibration of the *p*H gradients of the gels for *p*I determinations was achieved by the application of 15 μl of the Pharmacia Low pI Calibration Kit (*p*H 2.5–6.0) alongside the samples. Calibration curves were constructed from two IEF experiments with normal and abnormal plasmas by measuring the distances from the cathode that the *p*I markers had focused and plotting these against their corresponding *p*I values.

Gel Electrophoresis

Horizontal polyacrylamide gel electrophoresis (HPAGE) was performed in three different gel systems: *p*H 4.6, 8–10% T (Bell *et al.*, 1984); *p*H 7.9, 10.75% T; and linear gradient gels (HPAGGE) of 4–20% T, 10–20% T, and 10–17.5% T (Pollitt and Bell, 1983a). Buffer concentrations, gel dimensions, method of casting, and running conditions were as outlined by Pollitt and Bell (1983a) and Bell *et al.* (1984). Acid (*p*H 4.6) HPAGE was used not only for Pi typing but also as a second-dimension gel to obtain better resolution of the shifted proteins and to facilitate interpretation of the one-dimensional patterns.

Sample application and transfer of first-dimension IEF gel strips were essentially the same as those described by Pollitt and Bell (1983a). However, where *p*I determinations were performed in second-dimension IEF gels, strips 9 mm wide were cut from the first-dimension HPAGE gel (either *p*H 4.6 or *p*H 7.9) parallel to the direction of the electrophoresis and applied approximately 2 cm from the cathode and at right angles to the electric field of the focusing gel.

Molecular Mass Determinations

Molecular mass determinations were performed in linear gradient gels of *p*H 7.9, 4–20% T with the omission of 2-mercaptoethanol to maintain nondenaturing conditions (essential for the molecular weight marker proteins). Filter-paper strips saturated with a mixture of Pharmacia high and low

molecular weight markers were applied to the gel for calibration. The distances migrated by some of the commercial marker proteins and equine proteins such as postalbumin, esterase, and Pi proteins whose molecular masses had previously been determined by Pollitt and Bell (1983a) were measured. The results were analyzed using a program which fitted a parabola to a set of standards (Duggleby *et al.,* 1981). Calibration curves were calculated from two 2D HPAGGE experiments involving normal and abnormal plasmas.

Staining

HPAGE gels were stained for protein with the Coomassie brilliant blue G250–perchloric acid stain (Holbrook and Leaver, 1976), whereas IEF gels were protein-stained with Coomassie brilliant blue R250 (LKB application Note 1802). Esterase activity was visualized using α-naphthyl acetate and fast garnet GBC salt (Fisher and Scott, 1978) prior to protein staining. Inhibitory activities of the protease inhibitory proteins to bovine trypsin (Sigma Chemical Co.) and bovine chymotrypsin (Boehringer Mannheim) were determined by the method of Uriel and Berges (1968).

Neuraminidase Treatment

Selected plasma samples were treated with neuraminidase to determine the sialic acid contents of various Pi components in a manner similar to that described by Cox (1975) and Brown (1982) with the following modifications. Neuraminidase (from *Vibrio cholerae;* EC 3.2.1.18) of 1 U/ml activity was obtained from Calbiochem–Behring.

The assay consisted of 1.0 ml of plasma (lithium heparin anticoagulant) preincubated for 30 min at 37°C and the addition of 0.1 ml of the neuraminidase solution. The assay was performed in a circulating water bath (Julabo Model F20H) at 37°C in which the samples were kept agitated by the circulating water. Aliquots of 0.25 ml were removed at 15 min, 1 hr, and 6 hr, added to 0.05 ml of 0.12 M tetrasodium EDTA, and refrigerated (4°C). Following the removal of the third time point an additional 0.1 ml of neuraminidase solution was added and incubation allowed to proceed for a further 18 hr. At this time the solution was ultrafiltered by centrifugation in a Centricon 30 microconcentrator (30,000 MW cutoff membrane; Amicon) in a Beckman J-21C centrifuge (JA20 rotor) at 3000 *g* for 1 hr at 4°C and then in the recovery mode at 1000 *g* for 2 min at 4°C. This resulted in an approximately twofold concentration. The numbers of sialic acid residues were determined by counting the number of basic charge shifts exhibited by each of the proteins under study following 2D HPAGE (*p*H 3.5–6.0 IEF; *p*H

7.9, 10.75% T HPAGE; 10–20% T and 10–17.5% T HPAGGE) of the samples taken at various time points. The last sample represented some of the protein in its asialo form.

RESULTS

Pi F

The Pi F allele controls five major proteins with isoelectric points ranging from 3.74 to 4.27 and molecular masses of 55,500 to 69,000 (Pollitt and Bell, 1983a). The two most acidic proteins have ISO-DALT coordinates of 3.74; 69,000 and 3.92; 62,500 and inhibit only trypsin (Pollitt and Bell, 1983b). Neuraminidase treatment showed that these proteins contained eight and six sialic acid residues, respectively.

One case of abnormal expression of Pi F, designated F*, and in combination with Pi N has been found. The abnormal type was transmitted to two offspring, with Pi L being contributed by the other parent (Table I). On 1D acid HPAGE and 2D electrophoresis with acid HPAGE as the second dimension, the two affected proteins were absent from both the Pi F*L and the F*N patterns. However, it was observed that the two major acidic L proteins stained more intensely (open arrowheads in Fig. 1). Subsequent analysis by 2D electrophoresis with a *p*H 7.9, 10–17.5% T second dimension demonstrated basic shifts of the affected proteins to ISO-DALT coordinates of 3,905; 69,000 and 4.065; 64,000 (Fig. 2). These represented *p*I shifts of 0.165 and 0.145 for the most and second most acidic proteins, respectively.

No molecular mass differences were observed between the two states of expression of the acidic proteins with the exception of the second most acidic protein, which appeared to have increased in mass. This could be explained as a result of an error in the original M_r determination (Pollitt and Bell, 1983a), as the M_r of the second most acidic protein was recalculated as 63,500, 1000 heavier than the previously determined value. Based on this evidence, it is

Table I. Matings Showing the Allelic Inheritance of the Abnormal Types, Pi F* and S_1*

Mating type	Offspring
LL × F*N	2 F*L
LN × —	1 LS_1*
FL × LS_1*	1 FS_1*
FL × LS_1*	1 LS_1*

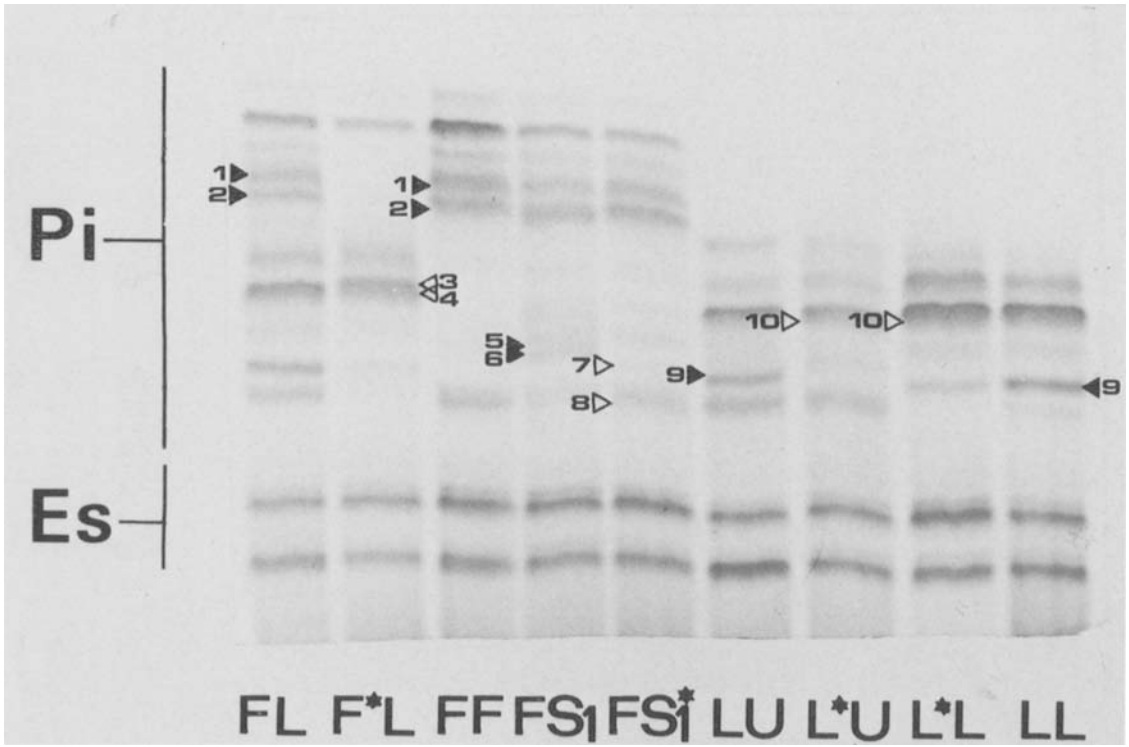

Fig. 1. One-dimensional *p*H 4.6 HPAGE gel patterns stained for protein and esterase and showing normally and abnormally expressed Pi alleles, F, L, and S_1. The abnormally expressed alleles are designated by asterisks. Filled and open arrowheads indicate the normal and shifted proteins, respectively. The anode is at the top of the photograph. The ISO-DALT coordinates for the numbered arrowed proteins are as follows: 1—3.92; 63,500; 2—3.74; 69,000; 3—4.065; 64,000; 4—3.905; 69,000; 5—4.18; 64,000; 6—3.98; 70,000; 7—4.23; 65,500; 8—4.03; 70,500; 9—4.38; 56,500; 10—4.315; 56,000.

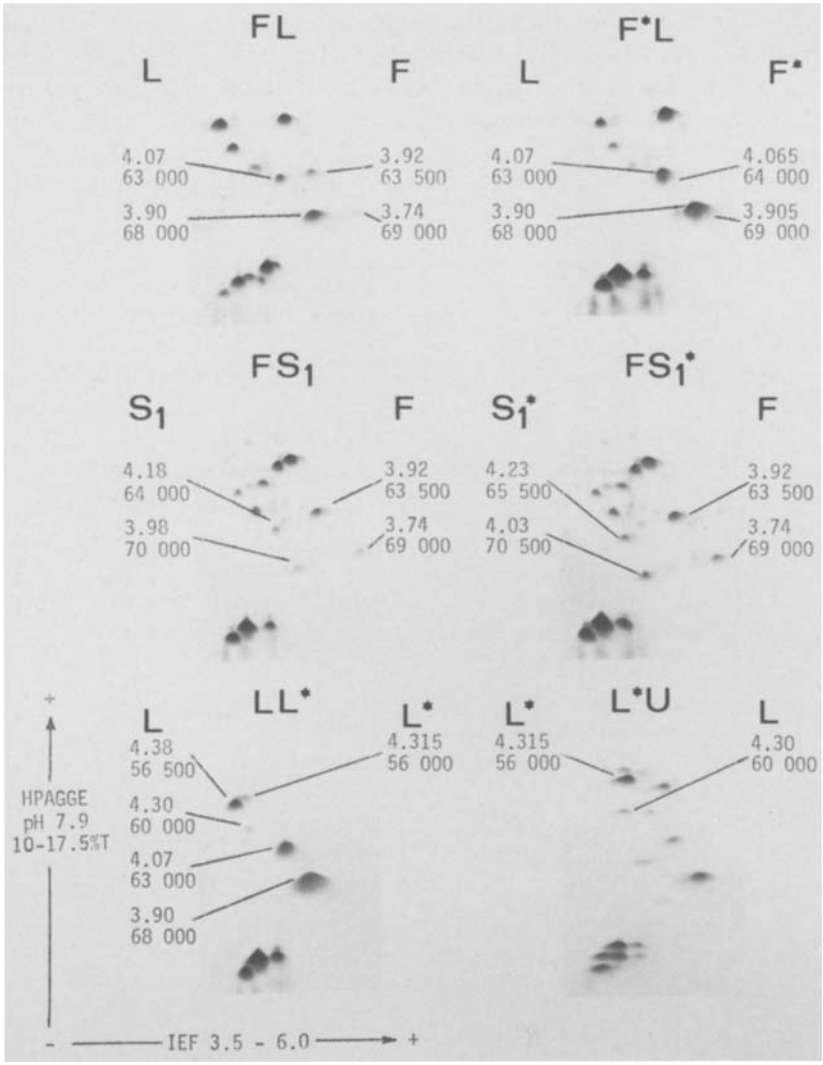

Fig. 2. Two-dimensional HPAGGE patterns (IEF *p*H 3.5–6.0 and *p*H 7.9, 10–17.5% T) of Pi types, FL, F*L, FS$_1^*$, LL*, and L*U, stained for protein and esterase. The Pi proteins of interest and the reference proteins are labeled with their ISO-DALT coordinates. The abnormally expressed alleles are designated by asterisks.

unlikely that this protein had increased in molecular mass in the abnormal type.

Neuraminidase treatments of the abnormal plasmas, Pi F*L and F*N, demonstrated that the most acidic protein contained six sialic acid residues, and the second most acidic, four residues. Therefore, both acidic proteins possessed two fewer sialic acid residues than their normal counterparts. The patterns suggested that the missing sialic acid residues were the first two to be removed, as the neuraminidase treatment charge shift patterns for the remaining residues appeared the same as those of the normal patterns.

Pi S_1

Pi S_1 is characterized by four major proteins with isoelectric points ranging from 3.98 to 4.25 and molecular masses of 56,000 to 70,000 (Pollitt and Bell, 1983a). As was the case for the abnormal expression of Pi F, the proteins of interest were the two most acidic of S_1, having ISO-DALT coordinates of 3.98; 70,000 and 4.18; 64,000. However, in contrast to the corresponding Pi F proteins, these proteins inhibit both trypsin and chymotrypsin (Pollitt and Bell 1983b). By neuraminidase treatment it was determined that the most acidic protein (*p*I 3.98) contained eight, and the other three major proteins, six (*p*I 4.18) and five (*p*I 4.10 and 4.25) sialic acid residues.

The abnormal expression, S_1, was found in the heterozygous state with Pi L and was transmitted to two offspring in combination with Pi F and L (Table I). On 1D acid HPAGE the loss of the cathodal bands in S_1^* was concomitant with the appearance of bands that were more cathodal (open arrowheads in Fig. 1). Analysis by 2D HPAGE (*p*H 3.5–6.0 and *p*H 7.9, 10–17.5% T) revealed basic shifts of 0.05 *p*H unit to ISO-DALT coordinates of 4.03; 70,500 and 4.23; 65,500 (Fig. 2).

Neuraminidase treatment revealed the loss of one sialic acid residue from both of the affected proteins. Figure 3 illustrates the basic charge shift patterns of the normal and abnormal S_1^*, exhibited the same general pattern as S_1 but the spots, number 4 of 3.98; 70,000 and number 2 of 4.18; 64,000, were absent in the abnormally expressed proteins. The abnormal proteins retained their inhibitory capacities for both chymotrypsin and trypsin.

Pi L

Four major proteins constitute the 2D pattern for Pi L with isoelectric points in the range of 3.90 to 4.38 and molecular masses of 56,500 to 68,000 (Pollitt and Bell, 1983a). The Pi L protein of interest is the most basic protein, with ISO-DALT coordinates of 4.38; 56,500. This protein inhibits both trypsin and chymotrypsin (Pollitt and Bell, 1983b). The numbers of sialic acid residues for

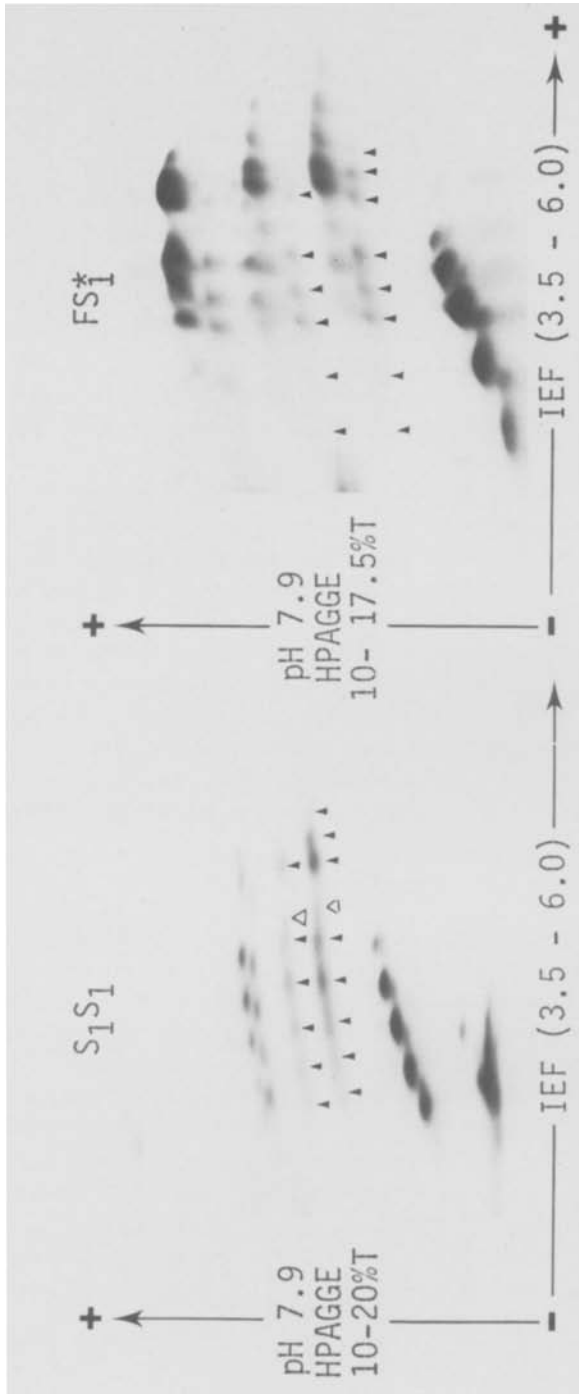

Fig. 3. Two-dimensional HPAGGE patterns (*p*H 3.5–6.0, *p*H 7.9, 10–17.5% T and 10–20% T) of neuraminidase-treated plasmas (24-hr time points) of Pi types, S_1S_1 and FS_1^*. Filled arrowheads show the original S_1 and S_1^* proteins and the proteins following the sequential removal of the sialic acid residues. The open arrowheads indicate the second and fourth spots, which are missing in the abnormal S_1 type.

the Pi L proteins were determined to be eight (*p*I 3.90), six (*p*I 4.07), three (*p*I 4.30), and four (*p*I 4.38).

An abnormal expression of Pi L, designated L*, was found in only 6 of a total of 83 offspring from the same stallion which also possessed the normal Pi L allele. The abnormal pattern was observed in combination with Pi G, L, N, and U, while the remaining 77 offspring inherited the normal allele from the sire. The stallion, Pi LL*, did not exhibit all the characteristics of the abnormal expression. This was reflected in a lower concentration of the shifted protein in comparison to that occurring in its offspring. The abnormally expressed allele, Pi L*, has been found to be inherited through two generations, with one of the stallion's affected offspring transmitting the abnormally expressed allele to one of four of its offspring.

On 1D acid HPAGE and 2D electrophoresis with an acid (*p*H 4.6) second dimension, the loss of the most cathodal Pi L zone in the abnormal type coincided with the appearance of an anodal zone of lower concentration but with the same inhibitory spectrum (open arrowhead in Fig. 1). After 2D electrophoresis the abnormal protein exhibited an acidic shift to ISO-DALT co-ordinates of 4.315; 56,000 (Fig. 2), representing an acidic *p*I shift of 0.065. Neuraminidase treatment demonstrated that this protein contained four sialic acid residues, as did the normal protein. The shifted protein had the same inhibitory spectra toward trypsin and chymotrypsin as the normal protein.

It is intesting to note that the protein with coordinates of 4.30; 60,000 was unaffected (Fig. 2). The use of the narrow-range IEF, 4.2–4.9, for the first dimension resulted in a splitting of the protein of interest into two very close spots which were evident in both the normal and the abnormal types. Whether or not this is an artifact of the carrier ampholytes has yet to be determined.

DISCUSSION

The simultaneous shifts of both acidic proteins, Pi F* and S_1^*, suggests that the corresponding normal proteins are related and derived from a single gene. It would appear that due to rate limitations of the posttranslational processing pathway, two forms of the protein that differ by one biantennary side chain, which has two terminal sialic acid residues, are released into plasma. The molecular mass difference between the two acidic proteins is approximately 6000, whereas a biantennary side chain accounts for only 3500–4000 M_r. The discrepancy in M_r may not be significant. Pellegrini *et al.* (1985) reported a difference in M_r of 3000 between the two acidic proteins, which they termed "isoforms." Incomplete glycosylation of one of the human plasminogen variants has been postulated to account for the two variants found in plasma (Powell and Castellino, 1983). Alternatively the difference between the two

normal proteins could be accounted for by proteolysis of a small terminal region which included a carbohydrate side chain.

All of the eight "Thoroughbred" Pi alleles have two acidic proteins that differ in *p*I and M_r by 0.15–0.20 *p*H unit and 5000 to 6000 daltons, respectively (Pollitt and Bell, 1983a). All of the 10 "new" alleles found in Standardbreds (Bell *et al.*, 1984) also have two acidic proteins that differ in *p*I and M_r by 0.13–0.17 *p*H unit and 4500 to 6000 daltons, respectively (Patterson and Bell, unpublished results). The finding of abnormal types which involved shifts of both acidic proteins supports the idea first suggested by Juneja *et al.* (1979) and supported by Pollitt and Bell (1983b) that these two proteins are related. This may be a general characteristic of the two most acidic protease inhibitory proteins in the horse. Pellegrini *et al.* (1985) separated two isoforms of a horse α_1-protease inhibitor which had M_r values of 50,500 to 59,000 and 53,000 to 62,000 daltons by SDS PAGE. This microheterogeneity was not observed by our nondenaturing techniques on native plasma and is most likely due to their method of purification.

The abnormal expression of Pi F appears to be simplest to explain, as both acidic proteins exhibited average basic *p*I shifts of 0.155 *p*H unit, which correspond to losses of two sialic acid residues (Jeppsson *et al.*, 1978). There were no changes in molecular mass. The alteration of carbohydrate content would appear to be the result of some change specific to the two acidic proteins and not a general posttranslational event modification (Elbein, 1984), as no abnormalities of either transferrin, group-specific component, or esterase were found in horses exhibiting the Pi F abnormality. The abnormal Pi F type may be the result of a single mutational event which prevents the normal processing of the biantennary side chain which is common to both acidic proteins, by the loss of some recognition signal for processing. This results in proteins of similar mass but possessing a side chain lacking sialic acid. Green (1982) showed that the incorporation of amino acid analogues interfered with the processing of the complex sialic acid side chain, resulting in a simple high mannose one. The Pc 1 Duarte brain polymorphism is similar to the Pi F abnormality in that it has been postulated that a single amino acid substitution results in a variant form showing the same three-spot pattern but all with equal basic shifts (Comings, 1979).

The abnormal expression of Pi S_1 is characterized by both acidic proteins being affected by losses of single sialic acid residues, basic charge shifts of 0.05 *p*H unit, and small increases in molecular masses of between 500 and 1500 daltons. The loss of the sialic acid residue would be expected to result in a decrease in M_r. However, a small increase was observed. Again, this change in molecular mass may not be significant, as it is only slightly greater than the standard error of the M_r determination (SE, $\pm$600 daltons). Lack of process-

ing of a carbohydrate side chain could result in a side chain with no terminal sialic acid residue. The reason for the alteration of the normal processing may be the same as that suggested for the abnormal Pi F type (amino acid substitution).

The absence of spots from the charge shift pattern of the neuraminidase-treated S_1^* plasma (Fig. 3) suggests that this is due to an inhibition of processing of a particular carbohydrate moiety which is present in both proteins. Sequential removal of the terminal sialic acid residues would occur in order of the ease of accessibility of the enzyme to these residues. The sialic acid residues lacking in the abnormal type correspond to the fourth spot of the 3.98; 70,000 protein and the second spot of the 4.18; 64,000 protein, these being the third and most accessible residues, respectively. These terminal residues would appear to be identical in the normal proteins. The difference in charge shift number further supports the theory that a rate-limiting process is responsible for the biantennary side-chain difference between the two proteins. Presumably this side chain would be the most accessible, as it is the last to be attached.

In contrast to the Pi F and S_1 abnormalities, that of Pi L does not involve any change in sialic content despite an acidic *p*I shift of 0.065 for the most basic protein. The M_r decrease of approximately 500 daltons is inconsistent with an extra sialic acid residue and probably does not represent a significant change in M_r. Possible explanations for these findings are an amino acid substitution resulting in a change in *p*I of the protein either directly or by alteration of the tertiary structure or a loss of an amino terminus.

The distorted segregation of Pi L* in the offspring (6 in 83) is difficult to explain at this stage. No explanation can be offered for the lower concentration of the shifted protein in the sire which is not reflected in the offspring possessing the abnormality. The fact that only one of the two more basic lower molecular mass proteins of Pi L is involved indicates that these two proteins are most likely encoded separately.

Studies of the abnormal expressions of Pi F,L, and S_1 have aided in the elucidation of the genetics of the equine Pi system. Originally the system was considered to be controlled by a single locus (Gahne, 1966; Braend, 1970) but subsequently a theory of two closely linked loci, Pi1 and Pi2, based on differences in M_r and inhibitory spectra was proposed (Juneja *et al.*, 1979). Our findings of both acidic proteins being affected in the Pi F and S_1 abnormalities and the work of Pellegrini *et al.* (1985) confirm the existence of the Pi2 locus (high molecular mass acidic proteins).

It is logical to assume that counterparts to the human protease inhibitors occur in horse plasma. Juneja *et al.* (1979) claimed on the basis of inhibitory activities and electrophoretic mobilities that the more basic, lower molecular mass proteins (designated Pi1) were the equivalent of the human α_1-Pi. Our

preliminary results with immunoblotting using antiserum to human α_1-antitrypsin demonstrated cross-reaction with the Pi1 proteins, thereby confirming the proposal of Juneja *et al.* (1979) that these correspond to the human α_1-antitrypsins. The fact that only one of the two major Pi1 proteins is shifted in the L abnormality infers that more than one locus is concerned with the genetic control of the Pi1 proteins. Human α_1-antichymotrypsin inhibits mainly cathepsin G and also chymotrypsin but neither trypsin nor elastase (Travis *et al.*, 1978a). It consists of various isoforms with isoelectric points of 4.5–5.1 and molecular masses of 58,000–68,000 (Daly and Hallinan, 1984; Laine and Hayem, 1981; Travis *et al.*, 1978b). Equine class III proteins which, according to their classification, are α_1-antichymotrypsin, have a M_r and *p*I of approximately 60,000 and 4.2, respectively. These proteins may well be the equine equivalent of human α_1-antichymotrypsin.

Equine antithrombin III, which was isolated by affinity chromatography and shown to have a molecular mass between 53,000–58,000, contains five or six sialic acid residues and cross-reacts with human antithrombin III antiserum (Kurachi *et al.*, 1976). Pellegrini *et al.* (1983) identified an α_2-antiprotease (62,000–71,000 M_r and five *p*I values in the range 5.2–5.8) in horse plasma as antithrombin III. It is not known which of the equine proteins in our study is antithrombin III. Characterization by immunoblotting is currently in progress. The heterogeneity described by Pellegrini *et al.* (1983) was not observed in this study and may be attributed to the loss of sialic acid residues during their isolation procedure.

Two cases of irregular transmission in the equine Pi system have been reported previously by Braend (1980). A stallion, Pi FW, and a mare, Pi FS, produced offspring which exhibited anomalous patterns, designated D_1 and D_2. These were characterized by minor variations in the Pi F patterns and both were transmitted as alleles. It has not been possible to determine whether our Pi F abnormality corresponds to Braend's D_1 or D_2.

Abnormal expressions of the bovine transferrin alleles have been reported (Spooner and Baxter, 1969). Losses of sialic acid residues from the two anodal proteins resulted in decreased mobilities to those of the two cathodal proteins. This was explained by a breed-specific (Hereford) recessive epistatic gene which affected the attachment of sialic acid to the anodal transferrin proteins.

Two of the major protease inhibitor loci, α_1-Pi and α_1-antichymotrypsin, are linked in humans (Rabin *et al.*,1985) but antithrombin III is located on a separate chromosome (Bock *et al.*, 1984). Using nucleic acid analysis, Hill *et al.* (1985) reported a close linkage between two serum protease inhibitor genes, Spi-1 and Spi-2, in both mice and rats. In the pig, four protease inhibitor loci, Pi1, Po1A, Po1B, and Pi2, are tightly linked (Gahne and Juneja, 1985). It is likely that a similar situation will occur in the horse, as the

multibanded Pi pattern, which presumably includes counterparts to the human Pi proteins, appears to be inherited as a single genetic unit.

Future studies will be directed toward the purification of the various components of the equine Pi system. Subsequent immunoblotting, peptide mapping, and amino acid sequencing will aid in the identification and further characterization of this interesting system.

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