Acta vet. scand. 1982, *23*, 592—602.

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GENETIC VARIATION OF THE EQUINE SERUM PROTEASE INHIBITOR SYSTEM Pi (Pr) CHARACTERIZED BY AN ENZYME BINDING STAINING TECHNIQUE AFTER STARCH GEL ELECTROPHORESIS

By

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BRAEND, MIKAEL: *Genetic variation of the equine serum protease inhibitor system Pi (Pr) characterized by an enzyme binding staining technique after starch gel electrophoresis.* Acta vet. scand. 1982, *23*, 592—602. — A modification of *Uriel & Berges* (1968) staining technique has been developed for starch gels. This method, which makes use of the Pi proteins ability to bind trypsin and chymotrypsin, allows for the recognition of the Pi zones which migrate into slower positions than originally described by *Braend* (1970). The Pi zones appear as white bands against a lilac background. Serum samples from 18 sire families selected according to the Pi type of the sires have been studied. Ten families were Norwegian Trotter, 8 were Warmblood Trotter (Standardbred). In each family 12 dam-offspring pairs were examined. In trypsin-treated gels the white zones usually correspond to those previously recognized by protein-staining. In addition, the products of the Pi^G, Pi^I, Pi^L and Pi^W alleles each had 1 distinct slow band, but in different positions. The products of the Pi^N and Pi^U alleles lacked slow zones. The Pi^S and Pi^T alleles differed with respect to the positions of their slow bands. A new allele Pi^{L_1} was identified. This has a slow band in a different position from that of the Pi^L allele. An allele indistinguishable from Pi^Z was recognized in Norwegian Trotter in which also a new allele temporarily called Pi^Y could be demonstrated. In chymotrypsin-treated gels the zone patterns of some of the allele products differed from those seen after trypsin-treatment.

horse; serum; protease inhibiting protein.

The polymorphic plasma protein system named Pr was first described by *Gahne* (1966). It was shown to be a protease inhibitor by *Ek* (1977). Using antigen antibody crossed electrophoresis *Ek* (1979) also extended *Braend*'s (1970) original Pr

system by the demonstration of slower zones for most of the alleles. The extension of the system was confirmed by *Scott* (1979) with the detection of the Pr^Z allele. *Pollitt & Bell* (1980) confirmed the protease inhibiting effect of the Pr zones using an acid polyacrylamide technique which clearly delineated a large number of zones within the Pr system. These authors suggested that the Pr system be renamed, the Pi system.

This change of name was also proposed by *Juneja et al.* (1979). Using a 2-dimensional technique involving alkaline polyacrylamide and acid agarose gel electrophoresis *Juneja et al.* interpreted the Pi system as being controlled by 2 linked genes. These were designated Pi 1 and Pi 2 with 6 and 5 alleles, respectively. *Matthews* (1979) using isoelectric focusing showed that most of the allele products appeared with similar patterns as those described by *Braend* (1970).

The present report describes further elucidation of the complex equine Pr protein system by the use of a staining technique based on that of *Uriel & Berges* (1968). Henceforth, the system will be referred to as the Pi system in this paper.

MATERIALS AND METHODS

Serum samples from 18 sire families have been studied. Ten were Norwegian Trotter, 8 were Warmblood Trotter (Standardbred). In each family 12 dam-offspring pairs were examined. The samples were received for service typing in 1974 and since then stored at —20°C. In addition, the 40 samples from the 1981 ISABR horse comparison test, plus 20 fresh samples from various horses have been examined.

The samples were subjected to starch gel electrophoresis according to the technique developed by *Braend* (1970) but with some modifications. Gel trays measured 21 cm × 12.5 cm × 0.45 cm. Gel buffer (pH 4.5) consisted of 52 ml 0.05 mol/l citric acid, 11 ml 0.2 mol/l tris (Sigma 7—9) and distilled water made to a volume of 400 ml (2 gels). Hydrolyzed Norwegian commercial potato starch or Connaught hydrolyzed potato starch was used at a concentration of 10 %. The mixture was heated on a cooking plate with constant mechanical stirring (R 15, Inter. Labor. App. GmbH. 7801 Dottingen, Germany). After cooking for about 15 min there was a distinct change in consistency. Heating and stirring was continued for another 2 min. Air bubbles were

sucked out by water suction for ½ min. The gels were covered with glass plates and were stored at room temperature for ½ h and then ½ h at +4°C.

The tray buffer (1 l per tray) contained 47 g H_3BO_3 (dissolved at 60°C) and 0.67 g Na OH per l. Tray buffers were used up to 10 runs. Insertion of samples, bridges, voltage, current and running time were as previously described (*Braend* 1970). For cooling, the gels were after ½ h run covered with cold water (appr. 100 ml) in a trough made by a sheet of thin polyethylen plastic having wooden bars (25 cm × 2 cm × 2 cm) underneath at each side...

For enzyme inhibition, staining and destaining the following solutions were used (for 4 gel halves):

Abbreviation	Description
PB	900 ml 5.5 % Na_2HPO_4 . $2H_2O$ and 50 ml 0.1 mol/l citric acid, pH 8.0.
Ne	1 g Neguvon (Metrifonat, Bayer) in 100 ml 16.5 % PB.
Tr	10 mg Trypsin (Sigma T-8253) in 200 ml PB.
Ch	10 mg Chymotrypsin (Sigma C-4129) in 200 ml 50 % PB.
N-A	100 mg N-Acetyl-DL-Phenylalanine β-Naphtyl Ester (Sigma A-7512) in 40 ml N, N-Dimethylformamide (Sigma D-4254).
o-D	200 mg o-Dianiside, tetrazotized (Sigma, D-3502) in 400 ml 16.5 % PB.
De	70 % of a solution containing 1 l metanol, 1 l water and 200 ml acetic acid.

After electrophoresis the gels were cut in two by the use of a thin nylon fishing line. Each half was placed in a plastic tray of size 18 cm × 14 cm × 3 cm. With a small painting brush the 4 gel halves were brushed over the area with the esterase zones with Ne (organophosphoric compound) which inhibits esterase activity. The two top parts were treated with freshly prepared Tr solution for ½ h at 37°C and ½ h at 56°C in a waterbath. The 2 bottom halves were treated with freshly prepared Ch solution for ½ h at 56°C in waterbath followed by 37°C in the incubator. After the enzyme solutions had been removed, the gels were washed with cold tap water and then 16.5 % PB.

Preparation of N-A was done after enzyme treatment had been initiated. Preparation of o-D however, was started 7 min

before it was mixed with N-A and then immediately poured on the washed gels. The gels were treated with this solution (lilac) for ½ h at 37°C. After the solution had been removed 10 % acetic acid was poured on to the gels and left over night. Then the solution was replaced by 100 ml De. After the protease inhibition zones had been destained and turned white the gels were wrapped in thin polyethylen plastic and stored at room temperature.

RESULTS

Fig. 1 A shows a photograph of a trypsin-treated and stained gel (Norwegian starch). Samples 1 to 7 are from the 1981 ISABR comparison test. No. 1 appears with fast bands corresponding to those seen after protein-staining. In addition, there are 2 slow zones, one belonging to the Pi L, the other to the Pi W type.

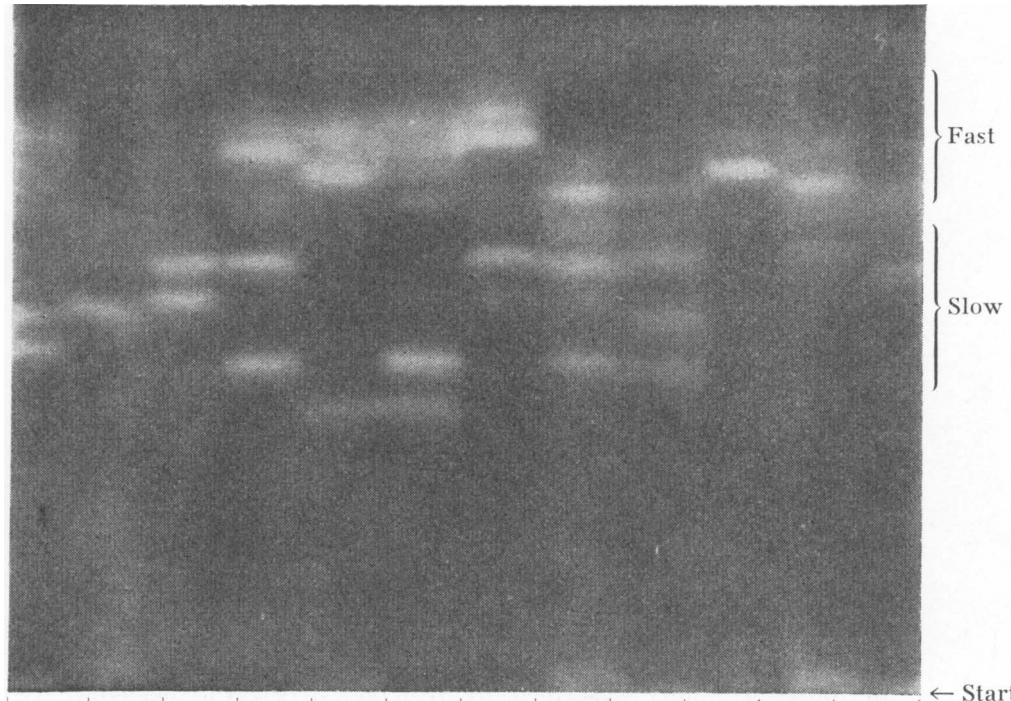
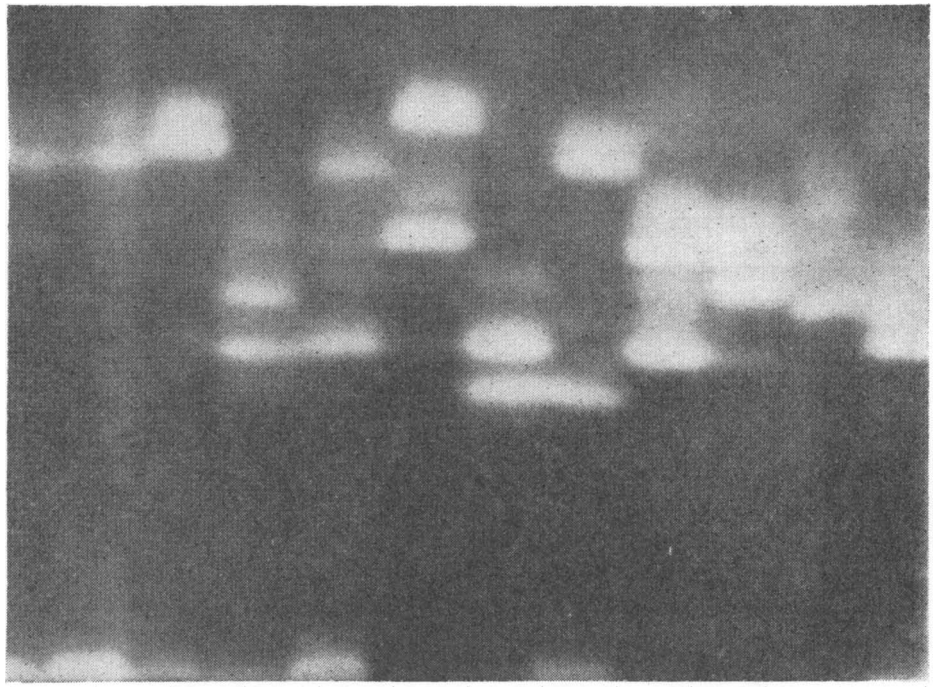


Figure 1 A. Photograph of a trypsin-treated gel.

Sample No. 2 (Pi UZ) shows 1 strong slow band slightly faster than the slow Pi W band and a very weak one ahead. These 2 are Pi Z bands. The Pi U band is also very weak on this photograph. Sample No. 3 does not show any fast zones, but the slow Pi G zone and a band which may belong to the product of an unknown allele and therefore called Pi X by *Braend* in the report on the 1981 comparison test (Unpublished). The next sample is Pi GL which in addition to the slow PiG and Pi L bands shows the fast Pi L bands, but no fast Pi G bands. In sample No. 5 there are weak fast Pi I bands, a slow Pi I band and 1 strong Pi N band. The fast Pi I zones are, however, stronger in sample No. 6 which also shows the fast Pi L bands as well as the slow bands of Pi I and Pi L. Sample No. 7 is a Pi FL_1. Only 1 strong and 1 weak fast Pi F band appear in this photograph and the slowest of the fast Pi L_1 bands is not visible. The Pi L_1 phenotype appears, however, with a slow band in about the same position as the slow Pi G band. Accordingly, the zone pattern of the Pi L_1 phenotype has 4 fast bands in about the same positions as the Pi L phenotype, but the second band is weaker than the second band of Pi L. Furthermore the Pi L_1 type has 1 strong band in a position about midway between the fast second band and the slow band of Pi L. The next 2 samples are from a dam-offspring pair (Norwegian Trotter). The sample from the dam shows the position of the Pi Y bands which are the products of a new allele called Pi^Y. The slowest of the Pi Y bands migrate at about the same speed as the slow Pi L band whereas the faster of the 2 slow Pi Y bands moves to the same position as the slow Pi L_1 zone. The offspring has received the allele giving the Pi Y zones from its dam and the one giving the Pi Z zones from the sire, indicating that the Pi Y phenotype consists of 2 major and 1 minor slow zones and a fast band in the same position as the fast Pi S band. Sample No. 10 is Pi N homozygous showing 2 fast bands, 1 strong and 1 weak. The last 2 samples (Norwegian Trotter) show the homozygous Pi S and Pi T phenotypes. The fast Pi S band has a weak one in front. The Pi T band is slightly slower, but also with a weak one ahead. These zones correspond to those originally described by *Braend* (1970). Furthermore, the Pi T has a slow zone in the same position as the slow Pi L_1 zone, whereas a weak slow zone of Pi S is faster.

Fig. 1 B is a photograph of a gel treated with chymotrypsin. The zones are generally stronger than those in the trypsin-treated



F i g u r e 1 B. Photograph of a chymotrypsin-treated gel.

gel. This is due to the technique. By varying the procedures and the buffers the zones may, after trypsin-treatment, appear much stronger. But then the fast bands are not always easily distinguishable within the patterns of individual alleles. Fig. 1 B shows the same slow zones as in Fig. 1 A. Those of samples 11 and 12, however, are weaker and not easily distinguishable. It can further be seen that the Pi G, Pi I, Pi L and Pi W types lack fast zones whereas Pi F has 1 intense fast zone. The Pi Y type has 1 slow band only.

Table 1 shows the distribution of paternal Pi types in the 18 sire families. The distribution is in agreement with the genetic theory. Accordingly, Pi^{L_1} and Pi^{Y} are additional alleles in the complex Pi system. The zone patterns, slow ones included, did, for the great majority of samples, appear as expected, according to the basic patterns demonstrated in Fig. 1 A and Fig. 1 B.

T a b l e 1. Paternal Pi phenotypes among offspring in 18 sire families.

Sire	Type	Offspring From sire	N.d.	Sire	Type	Offspring From sire	N.d.
1	NY	6N, 6Y		10	NZ	5N, 7Z	
2	IL_1	6I, $6L_1$		11	NU	10N, 2U	
3	FL	7F, 5L		12	LU	4L, 7U	1
4	LN	6L, 6N		13	LL_1	6L, $6L_1$	
5	FT	5F, 5T	2	14	IL	6I, 6L	
6	FN	5F, 7N		15	LN	4L, 8N	
7	TT	12T		16	LS	2L, 8S	2
8	SS	12S		17	UU	12U	
9	NT	8N, 4T		18	UU	12U	

N.d.: Not determinable; i.e., because of similarities with dam's phenotype, the offspring could have either of the two possible alleles.

Among the Norwegian Trotters, however, 1 dam-offspring pair and 2 mares previously typed as SS (3) and NL all had a band slower than the slowest L band but faster than the slow Pi I band. Among the Warmblood Trotters 4 animals showed zone patterns which did not fit the basic zone patterns. These exceptions will be dealt with in a following study. In this connection it should be mentioned that in samples which have been repeatedly frozen and thawed inhibition zones appear near the insertion points. In Fig. 1 B such zones are clearly apparent for samples Nos. 5, 8, 10, 11 and 12. These zones may represent aggregates of altered Pi molecules and could correspond to the aggregate forms which were suggested to be dimers by *Juneja et al.* (1979). In such samples the fast zones are less intense than in fresh samples.

DISCUSSION

Most of the fast allele products show the same number of fast major and minor bands as appear after protein staining (*Braend* 1970). They also agree with the peak patterns as described by *Ek* (1979) with the use of antigen antibody crossed electrophoresis, except for some of the slow zones. Thus the Pi^N and Pi^U allele has each 1 major fast band only with the enzyme binding technique, whereas after antigen antibody crossed electrophoresis they also appear with 1 and 2 major slower peaks, respectively (*Ek* 1979, *Ek & Braend* 1982). Accordingly, the allele product may after trypsin treatment be as many as 5 (Pi L

as an example). In this connection it has to be mentioned that additional slow minor bands may be recognized in some of the allele products, particularly if subjected to more intense enzyme treatment.

Juneja et al. (1979) reported the individual allele product to be 1 major and 1 minor fraction in the Pi 2 system, whereas in the Pi 1 system it could either be 2 or 3 fractions. Thus their Pi 1 type designated as E had 1 major and 2 minor fractions. The frequency of its controlling allele in Swedish Trotter, a breed which is very similar to Warmblood Trotter in Norway, strongly indicates that it corresponds to Pi U in the present study. On the other hand their type S in the Pi 2 system also corresponds to Pi U as judged by their gene frequencies in the 2 Swedish Trotter breeds. Consequently, a horse being homozygous Pi U would have 5 fractions and the phenotype Pi 1 EE, Pi 2 SS with the technique of *Juneja et al.* (1979).

Pollitt & Bell (1980) reported the products of 5 alleles to have 5 zones each, whereas 3 others had 4. Not all zones showed trypsin inhibition, however. The number of major and minor bands differed between alleles. There were also other differences when compared to the starch gel method. Thus the Pi F pattern was less intense than Pi F in starch gels. The Pi N phenotype showed minor zones more anodal than any of the Pi L zones. Whether their subdivision of the Pi S phenotype into Pi S_1 and Pi S_2 corresponds to the Pi S and Pi T needs to be determined in direct comparison tests.

From the above it follows that there are pronounced differences between the starch gel (*Braend* 1970) and the polyacrylamide methods (*Pollitt & Bell* 1980) in respect of number, position and intensity of zones. The use of polyacrylamide is advantageous since the gels are more reproducible. The resolution of starch gels may vary. There may be differences between batches even for commercially available hydrolyzed starch. In this connection it should be mentioned that a certain batch of Connaught starch gave less separation between the fast and the slow zones than the Norwegian hydrolyzed starch prepared in our laboratory. This may be remedied by changes in the procedures for making the gels, for instance with regard to time for cooking and the pH of buffers. On the other hand, the polyacrylamide method is far more complex and involves the use of quite a number of toxic substances.

The 2-dimensional method of *Juneja et al.* (1979) differs fundamentally from the 2 methods discussed above. Their first dimension separation takes place at pH 5.4. This does not give such a pronounced separation into zones as with starch gels of pH 4.8, which is in agreement with the results of *Ek* (1979). He showed that in starch gels of pH 5.4 there is very limited separation into Pr (Pi) zones. The second dimension separation of *Juneja et al.* (1979) takes place in agarose gels of pH 9.0. This results in further distinction between zones of their Pi 1 system. More important though, is the pronounced separation which is obtained between zones of their 2 systems and for zones within the Pi 2 system.

Whether the technique developed in the present studies will be used extensively for routine purposes remains to be seen. It is certainly an improvement of *Braend*'s (1970) method which increased the 4 Pr alleles (*Gahne* 1966) to eight. Later *Scott* (1977, 1979) reported the occurrence of the Pi^G and Pi^Z alleles and *Braend* (1980) the rare mutant alleles Pi^{D_1} and Pi^{D_2}. An allele called Pi^M by *Scott* is indicated in Polish horses, which were investigated in the 1979 ISABR comparison test. A similar allele has been recognized in Italian horses (*Lubas et al.* 1982). Consequently, if the 2 new ones of the present report are included, the following alleles have been reported: Pi^{D_1}, Pi^{D_2}, Pi^F, Pi^G, Pi^I, Pi^L, Pi^{L_1}, Pi^M, Pi^N, Pi^S, Pi^T, Pi^U, Pi^W, Pi^Z and Pi^Y. Whether Pi^{S_2} (*Pollitt & Bell* 1980, *Lubas et al.* 1982) is additional or the same as Pi^T remains to bee seen. But an allele different from those mentioned above was strongly indicated in the 1981 ISABR comparison test. It was called Z_2 or U_3 by those workers who gave it a symbol. In addition comes a possible allele giving the product X.

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SAMMENDRAG

Genetisk variasjon av hestens proteasehemmende Pi (Pr) system, bestemt ved hjelp av stivelsesgel elektroforese og en enzymbindende fargeteknikk.

En modifikasjon av *Uriel & Berges'* (1968) fargemetode er blitt utviklet til bruk for stivelsesgel. Denne metode beror på at Pi proteinene binder trypsin og chymotrypsin. Den muliggjør bestemmelse av Pi soner som vandrer saktere enn de som opprinnelig ble bestemt av *Brænd* (1970). Pi sonene viser seg som hvite bånd mot en lilla bakgrunn. Serumprøver utvalgt etter fedrenes Pi typer er blitt undersøkt for ialt 18 familier, 10 av norsk traver, 8 av varmblod traver. Hver familie bestod av 12 mor-avkom par. I geler som er behandlet med trypsin vil de hvite soner vanligvis tilsvare dem som sees etter proteinfarging. I tillegg viste produktene av *Pi^G*, *Pi^I*, *Pi^L* og *Pi^W* allelene et saktere, tydelig bånd hver, men i forskjellige posisjoner.

Slike sakte bånd manglet hos produktene til Pi^N og Pi^U allelene. Allelene Pi^S og Pi^T var forskjellige med hensyn til sakte bånd. En ny allel Pi^{L_1} er blitt påvist. Denne har et sakte bånd i en annen posisjon enn Pi^L allelen. En allel som synes å tilsvare Pi^Z ble funnet hos norsk traver, hvor også en ny allel, foreløpig kalt Pi^Y er blitt påvist. I geler behandlet med chymotrypsin viste sonemønstrene seg for noen av allelproduktene å være forskjellige fra dem som sees etter trypsinbehandling.

(Received September 27, 1982).

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