Protease Inhibitor (Pi) Phenotyping in Light Breed Horses on Isoelectric Focusing Using Urea-Polyacrylamide Gel

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Abstract Isoelectric focusing (IEF) using polyacrylamide gel containing urea (Urea-PAG) was used for the characterization of equine protease inhibitor (Pi) types in light breed horses to solve the following problems in conventional IEF and two-dimensional (2-D) electrophoresis: typing complexity due to high heterogeneity of *Pi* system and overlapping of *Pi* and esterase (*Es*) components on IEF, operational complexity and time-consumption on 2-D. The Urea-PAG IEF method had high resolution capacity, clearly distinguishing at least 10 types frequently observed in light breed horses. *Es* components were not detected on Urea-PAG. There was little complexity of *Pi* typing as shown by these results, and thus the typing procedure is simpler and more rapid than conventional IEF and 2-D. The present method should thus prove quite adequate for exact classification of *Pi* system in light breed horses.

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The equine protease inhibitor (*Pi*) system is the most polymorphic genetic among horse blood typing systems, and at least 18 alleles have been assigned by this system following the International Society for Animal Genetics (ISAG) Workshop discussion for the 1993 Horse Comparison Test (HCT). The *Pi* system is considered a very useful blood typing marker for identification and parentage testing owing to its highly polymorphic nature. Some informative typing techniques have thus been established and used for the classification of *Pi* alleles^{1,3-6)}.

The horizontal isoelectric focusing (IEF)

method reported by Yokohama and Mogi¹²⁾ is a very useful typing technique for the *Pi* system and is routinely used as a conventional method in horse blood typing. However, this method has complexity on *Pi* system because *Pi* protein has high heterogeneity and is detected at the same isoelectric point (pI) region where the F band of esterase (*Es*) system is located. Yokohama *et al.*^{13,14)} adopted the microscale multisample two dimensional electrophoresis (2-D) method to solve these problems. This 2-D technique separated *Pi* components from *Es* components and characterized the respective *Pi* types in light breed horses. It is thus

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routinely used as a confirmative step for typing when classification of Pi types on the IEF testing is complicated. However, there is an operational difficulty in this method in contrast to horizontal IEF, causing it to be time-consuming. To solve this problem and establish a simpler and more rapid routine typing, the horizontal IEF method should have high resolution capacity and the capacity to separate Pi and Es components. Therefore, in the present study, polyacrylamide gel containing urea was used for electrofocusing as a novel technique, and the capacity to separate Pi and Es components by denaturation of urea was examined. Potentiality for the resolution and repeatability was also assessed.

Specifically, the exact classification of *Pi* alleles in light breed horses has an important purpose for blood typing service as a part of their pedigree registration. The following *Pi* alleles, *Pi^F*, *Pi^G*, *Pi^I*, *Pi^L*, *Pi^M*, *Pi^N*, *Pi^S*, *Pi^{Sl}*, *Pi^U* and *Pi^{L1}* (*Pi^{Sl}* and *Pi^{L1}* alleles have been identified and assigned to ISAG nomenclatural *Pi^T* and *Pi^{L2}* alleles, respectively), were frequently observed in light breed horses as reported previously¹²⁾. Therefore, the usefulness of the present technique for routine typing of these 10 types in light breed horses is discussed.

Materials and Methods

Samples :

Serum samples were obtained from 950 horses consisting of 601 Thoroughbreds and 349 Anglo-Arabs whose Pi phenotypes had been determined by conventional IEF or 2-D and classified into 55 phenotypes formed from 10 types, namely F, G, I, L, M, N, S, T, U and L₂. Reducing treatment for samples was performed with 0.25 M dithiothreitol and 10 mM iodoacetamide at a 2 : 1 : 2 ratio for 1 hour at 37°C or overnight at 4°C as described by Yokohama and Mogi¹²⁾ with slight modification.

Electrofocusing :

Electrofocusing was carried out using a thin

layer urea-polyacrylamide gel (Urea-PAG). Because the composition of the gel for hemoglobin typing reported by Yokohama and Mogi¹⁰⁾ is simpler than that of the conventional gel for Pi typing, Urea-PAG was prepared in this study using the gel for hemoglobin typing with the following modifications : 0.75 ml SERVLYT (SERVA) AG 4-5 was used as the carrier ampholyte, 7.5 ml 8 M urea was used instead of distilled water and 30 μ l TEMED were added to the stock solution per gel plate (140 mm $\times$ 120 mm $\times$ 1 mm). 1 M H₃PO₄ and 1 N NaOH were used as anodal and cathodal buffers, respectively. The gel was electrophoresed initially at 350 V (3 W) and last at 1200 V for 3 hours.

Detection and identification :

The detection of serum proteins separated by Urea-PAG was carried out by the CBB-R250 staining method as reported previously¹¹⁾. Identification of Pi components on Urea-PAG was done based on a comparison with known Pi types determined by conventional IEF or 2-D testing. The inhibition of bovine trypsin and chymotrypsin for the identification of Pi components was done as described by Pollitt and Bell⁸⁾. The procedure for detecting Es activity was the same as that of Gahne²⁾. A1B glycoprotein (A1B) components, i.e., Post albumin (Pa) or Xk components, were identified by comparing the present variants with A1B types obtained by conventional IEF which could simultaneously detect variants as reported¹²⁾.

Calibration of pI on Urea-PAG :

After electrofocusing, 24 aliquot gel fragments obtained from Urea-PAG were dissolved in 5 ml distilled water. pI on Urea-PAG was determined based on calculated pH of the solutions.

Results

Fig. 1 shows electrophoretic patterns on Urea-PAG of serum proteins including 10 Pi types determined by the conventional tech-

Fig. 1. The respective electrophoretic patterns of 10 basic *Pi* types (F, G, I, L, M, N, S, T, U and L₂) in light breed horses using Urea-PAG.

nique. These visible patterns were separated into two regions, one consisting of multiple bands and the other, minor bands, the same as by conventional method. No inhibition of trypsin and chymotrypsin for these detected bands was observed, and consequently the *Pi* components could not be identified by this method. However, typing results of the multiple bands on Urea-PAG were completely consistent with those of the *Pi* system as determined by conventional IEF and 2-D testing, using 950 samples. These bands were thus considered *Pi* components (*Pi* in Fig. 1). The electrophoretic behavior of the minor bands was identical to that of A1B components in conventional IEF.

The bands indicating *Pi* components on Urea-PAG were sharply separated and respective *Pi* types were quite distinguishable. All heterozygous types formed from 10 types could be distinctly seen. The resolution results of the bands showed high repeatability.

No *Es* components were detected within the region of *Pi* components by the present method

Fig. 2. A comparison of electrophoretic behaviors of *Pi* and *Es* components on conventional IEF and Urea-PAG IEF. The respective phenotypes in *Pi* and *Es* system are shown: (F) in *Es* system indicates the phenotype including F type, and the asterisked bands on the conventional gel indicate the F bands in *Es* system.

(Fig. 2). This component could not be visualized by CBB staining nor could its activity be confirmed at all using Urea-PAG.

Although the regional patterns of *Pi* and A1B components on Urea-PAG apparently resembled those of the conventional gel as mentioned above, each estimated pI region of *Pi* and A1B components was higher than those determined using the conventional gel. The former was from 5.1 (at the upper band of F type) to 6.0 (at the bottom band of I type) and the latter, 6.1 (Fig. 1).

Discussion

By the present method, the serum proteins, *Pi* and A1B components, were detected in a higher pI region even though Yokohama *et al.*⁹⁾ reported they could be observed within the pI

region from 3.5 to 5.2. Estimated pI of Pi components on Urea-PAG clearly differed from those from 3.74 to 4.43 reported by Pollitt and Bell⁷⁾. In contrast, pI regions of Pi and A1B components were identical to previous results when a gel with the same composition as that of Urea-PAG except urea (non Urea-PAG) was used. Thus possibly separated serum proteins using Urea-PAG are at least denatured by urea. It then follows that negative response to the inhibition of trypsin and chymotrypsin is due to denaturation of Pi components. Denaturation by urea may also influence Es components since they could not be visualized and their activity was not observed on Urea-PAG. In fact, they were clearly detected within the Pi region and activity was observed on non Urea-PAG.

Although detected proteins using Urea-PAG should be regarded as denatured, as for the phenotyping of Pi system, the present method is recognized as having high capacity to distinguish at least 55 phenotypes consisting of 10 Pi types in light breed horses because of the high resolution capacity. In the conventional method, S and T types were particularly difficult to distinguish when they formed heterozygotes with other Pi types because pI of the bands on respective types were very close to those of certain bands found in other Pi types^{13,14)}. Additional difficulty in typing was observed when Es components were detected within the detected region of Pi components since pI of bands on S and T types were similar to that of the F band in the Es system (see Fig. 2). However, on Urea-PAG, both S and T types could be clearly distinguished and Es components fortunately were not visualized by denaturation using urea. Thus the above cited difficulty in typing is no longer present even if S and T types are heterozygous with any other type. Therefore, by the Urea-PAG IEF method, it should be possible to classify Pi phenotypes distinctly in spite of high heterogeneity. Also it is suggested that 2-D testing as

a confirmative step is not necessary in Pi routine typing when Urea-PAG IEF is used. This method should thus prove to be a simple and rapid routine means for exact phenotyping in the Pi system for light breed horses.

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