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COMMON AND SPECIES-SPECIFIC SERUM ESTERASES OF EQUIDAE

I. HORSE AND DONKEY

MARIE KAMINSKI
With the technical collaboration of MICHÈLE SYKIOTIS
Laboratoire d'Enzymologie du C.N.R.S., 91, Gif-sur-Yvette (France)

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SUMMARY

1. Horse and donkey serum esterases were compared using histochemical and immunological criteria in starch and agar gel electrophoresis, immunoelectrophoresis and immunodiffusion.

2. One out of several lipoprotein esterases shared by the two species, designated α-Q esterase owing to its variable migration rate in agar electrophoresis, migrates faster than albumin in agar but slower in starch. The antigen–antibody complex formed with either homologous or heterologous antibodies is enzymatically active; it hydrolyzes β- but not α-naphthyl acetates. These esterases are antigenically identical in horse and donkey; the corresponding antibodies are present in both anti-horse and anti-donkey rabbit antisera.

3. A non-lipoprotein esterase of α_1 location is found in horse sera exclusively. It migrates slower than albumin in agar and in starch; the antigen–antibody complex is enzymatically active, and it hydrolyzes both α- and β-naphthyl acetates. The corresponding antibodies are present only in anti-horse antisera, rabbit or donkey.

INTRODUCTION

Several differences, serological or based on electrophoretic polymorphism, were shown between horse and donkey blood components, such as erythrocyte antigens[1,2], phenotypes of transferrin[3,4], albumin[4] and hemoglobin[4–6]. On the other hand, the antigenic identity of several analogous serum proteins was also shown[2,7].

The investigation of serum esterases was undertaken as another expression of phylogenetical relationship. Thus, a horse esterase, fast migrating in starch gel, was not detected in donkey[8]. The examination of about 100 donkey and over 300 horse sera, belonging to ten breeds[9,10], led to definition of five esterase activity zones, observed in starch gel. Out of three most frequent components, E_2, E_4 and E_5, the former two were

found in all donkey sera, while E_5 was always absent. However, taking into account the variable migration rate of the E_4, it was felt that the histochemical identification of esterases in starch gel should be completed by another approach.

In the present paper, the common and different esterases of two species were detected and identified by means of immunoelectrophoresis. Their antigenic properties were examined in homologous and heterologous reactions, and they were isolated from starch gel and analyzed in agar, using the same histochemical reactions.

MATERIALS AND METHODS

Horse and donkey sera

A hundred sera of various breeds (for details see ref. 10) as well as sera from successive bleedings of one animal were tested. The samples were either fresh or stored frozen for periods of a few months up to 6 years.

Immunosera

Rabbits were hyperimmunized with whole horse or donkey serum; they received either four weekly injections of 0.5–1 ml during 3 weeks or three injections of 1 ml of serum mixed with equal volume of Freund complete adjuvant at 3-week intervals and were bled 2 or 3 weeks afterwards. The sera were pooled; three resulting antisera anti-horse and three anti-donkey were used. In addition, a donkey anti-horse antiserum was also available.

Electrophoresis in agar gel

New Zealand agar at 0.75% in veronal buffer of 0.0025 ionic strength (pH 8.2) was used; with 5 V/cm in 3 h, the center of albumin spot migrates 5.5 cm.

The immunoelectrophoretic comparisons of horse and donkey sera were performed on the same slide, using both anti-horse and anti-donkey antisera for each antigen.

The complete characterization of esterases was achieved either by carrying out different histochemical reactions on duplicate slides or successively on the same slide.

Characterization of esterases

It was performed according to URIEL[11,12], following the routine method[13]; the substrates and dye couplers used were: α-naphthyl acetate with Fast Blue RR or Fast Violet B; β-naphthyl acetate with Diazo Blue B, Garnet GBC or Fast Violet B; naphthol As-D acetate with Fast Violet B, Garnet GBC or Diazo Blue B; indoxyl acetate.

β-Carbonaphthoxycholine iodide was used only in simple electrophoresis, as it was not hydrolyzed by any of the antigen–antibody complexes formed.

α- or β-Naphthyl butyrates or propionates did not yield significantly different results than those of acetates.

The inhibition assays were carried out by incubation of slides in DFP (diisopropylfluorophosphate), prostigmine (Roche), p-chloromercuribenzoate or quinine·HCl for 1 h.

Lipids were revealed with Lipid Crimson and proteins with Amido Black.

Starch gel electrophoresis and elution of esterase fractions

The discontinuous buffer system Tris–citrate–borate (pH 8.7) was used. (A = 8.76 g Tris *plus* 1.33 g citric acid per l; B = 11.9 g boric acid *plus* 1.2 g LiOH per l; 5.5 vol. of A *plus* 1 vol. of B for the gel and B alone for the troughs.) Under 8 V/cm in 6 h the center of albumin spot migrates 8 cm. The whole layer was 9 mm thick; one 3-mm slice was revealed for esterase with β-naphthyl acetate. As soon as the color appeared, bluish for E_4 and purple for E_5, the corresponding areas were located on the unstained slab and their central parts were cut off. The right localization of esterase spots was controlled by incubation in substrate; only weakly colored halos remained around the holes. The eluates were obtained by squashing of gel pieces in buffer, thoroughly mixing and centrifuging; eluates from several parallel runs were added.

RESULTS

Agar histoelectrophoretic comparison of donkey and horse esterases

Fig. 1 shows that common and different components cannot be determined using a single reaction. Four zones were differentiated: 1, corresponding to the low-density lipoprotein; 2, corresponding to the pseudocholinesterase; 3, a non-lipoprotein esterase resistant to prostigmin, NaF and quinine, inhibited by 0.01 M DFP; this very active zone was found in horse sera only; 4, corresponding to the high-density lipoprotein, not active on α- or indoxyl acetates, characterized by a variable electrophoretic location between α and ϱ.

The α-naphthyl acetate appeared as selectively hydrolyzed by the Zone 3.

Summing up, an α_1 non-lipoprotein esterase was observed in horse sera only.

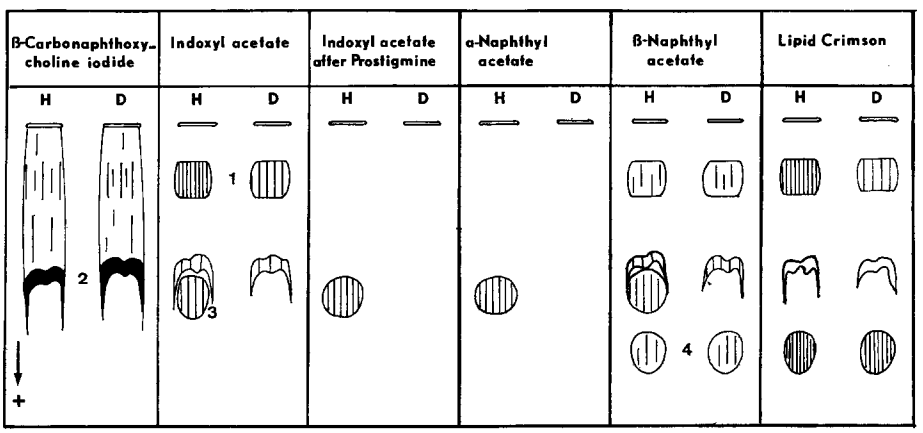

Fig. 1. Schematic representation of esterase-active and lipid zones observed in agar electrophoresis of horse (H) and donkey (D) sera, using different histochemical reactions. No. 1–4 refer to components described in the text.

Immunohistoelectrophoretical comparison of horse and donkey esterases

Proceeding from the origin towards the anode, eight esterase-active lines were observed (Fig. 2).

(a) Three β_1-lipomacroglobulins, neither migrating nor diffusing under the

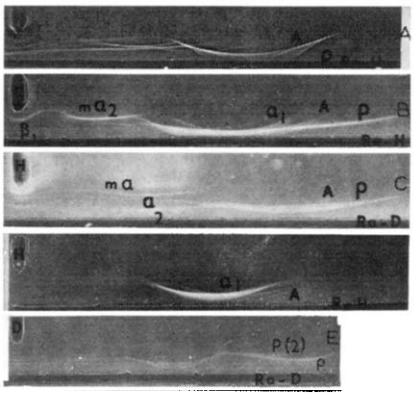

Fig. 2. Immunohistoelectrophoretical analysis of horse (H) and donkey (D) esterases; demonstration of eight different components. Greek letters design esterase-activity lines; A, albumin which, although unstained, is easily discernible on wet or dry slides; Ra-H and Ra-D, anti-horse and anti-donkey rabbit antisera, respectively (anti-donkey antisera on Slides C and E are different). Note the variable location of the line designed $m\alpha_2$. A. Visible precipitation lines (contact print). B. The same slide after esterase revelation. D. A parallel, duplicate slide. Slides B, C and E were revealed by β-naphthyl acetate, Slide D with α-naphthyl acetate.

experimental conditions, appearing as little parentheses around the reservoir; designated β_1-esterases.

(b) One α-lipoglobulin, varying between slow α_2 and α_0. This line is bordering a diffuse lipidic trail and designated macro-α.

(c) One α_2-lipoglobulin, diffusing more than previous components; designated α_2-esterase. Although present in both horse and donkey sera, it is revealed only with rabbit anti-donkey, and not with anti-horse antisera.

(d) One α_1-globulin, designated α_1-esterase.

(e) Two fast-migrating components: one, a lipoprotein, designated α–ϱ-esterase; the second, a short arched line, observed with one antiserum only; designated ϱ_2-esterase.

These components were not all observed in each sample examined and their recognition required constant comparison with control sera as well as the use of various histochemical reactions. Moreover, because of the variable electrophoretic migration of some of them, their identification was sometimes troublesome.

On the other hand, certain lines joined as if they were antigenically identical. For example, one of the β_1 coalesced with that of macro-α line when this latter was located in α_2 zone (Fig. 2B); also, the α_2-esterase fused with α–ϱ (Fig. 2C). Thus it appears that essentially the lipoprotein esterases showed this tendency.

The esterases listed in (a), (b) and (c) were found in horse and donkey sera, having similar properties.

The present work reports investigations on two components: α_1-esterase and α–ϱ-esterase.

Esterases detected in homologous reactions

(1) *Horse serum with rabbit anti-horse*

α_1-*Esterase*. Following incubation in either α- or β-naphthyl, naphthol As-D or

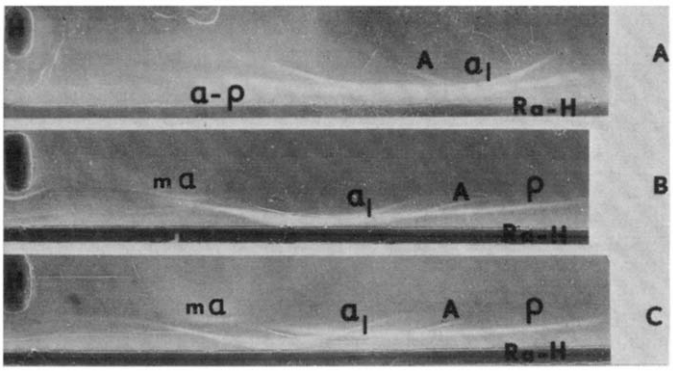

Fig. 3. Demonstration of variable location of ϱ-esterase. A. Longer than usual migration time (6 h) did not prevent the location of ϱ-esterase in α_1 zone, for a fresh serum sample. B. Cathodal extremities of the two lines are superimposed; only anodal ends are separate. C. The two lines cross each other, sample stored for 10 months. Abbreviations: see legend to Fig. 2.

indoxyl acetates (Figs. 2B and 2D), a regularly arcshaped line appears in α_1 zone, crossing the line of albumin (unstained). Although the color reaction was intense, the corresponding precipitation line was not discernible on unstained slides.

α–ϱ-Esterase. This line, as indicated by its name, is found in the α_1 zone (Fig. 3A), most often in the α_0 region with a more or less elongated anodic tail (Fig. 3B), or in the anodic region (Fig. 3C). Such variation is related to the duration of storage: the older the sample, the faster the migration (similar observations were reported for avian[13] and human sera[15,16]. Consequently, the α_1 and α–ϱ lines are often almost undistinguishable from each other. Despite this difficulty, the characterization of α–ϱ esterase and its differentiation from α_1-esterase were achieved in three ways: (a) owing to their substrate specificity; α–ϱ is revealed by β-naphthyl acetate, but not by α-naphthyl acetate, indoxyl acetate or naphthol As-D acetate (Figs. 2B and 2D), whereas α_1 is active on all of them; (b) α–ϱ is stained by Lipid Crimson, while α_1 is not; (c) the two lines cross, indicating their different antigenic specificities (Fig. 3C).

(2) *Donkey serum with rabbit anti-donkey (Figs. 4A and 4C).* The α–ϱ esterase is revealed with β-naphthyl acetate; likewise for horse component, it reacts with Lipid Crimson. No α_1 line could be detected nor was any activity revealed with α-naphthyl acetate, indoxyl acetate or naphthol As-D acetate.

Esterases detected in heterologous reactions

(1) *Horse serum with rabbit anti-donkey.* As shown in Figs. 4B and 4D, the only esterase detectable was α–ϱ, revealed with β-naphthyl acetate.

(2) *Donkey serum with rabbit anti-horse.* In keeping with previous results, the only esterase revealed was again the α–ϱ (Figs. 4A and 4C).

Esterases detected with donkey anti-horse

As seen in Fig. 4E, the only esterase detected is, this time, the α_1, formed by the horse serum.

Summing up, the α–ϱ-esterase, present in both horse and donkey sera, can be demonstrated after immunological reaction with either homologous or heterologous antisera; the α_1-esterase, present in horse and absent in donkey serum, can be demonstrated exclusively with homologous antisera.

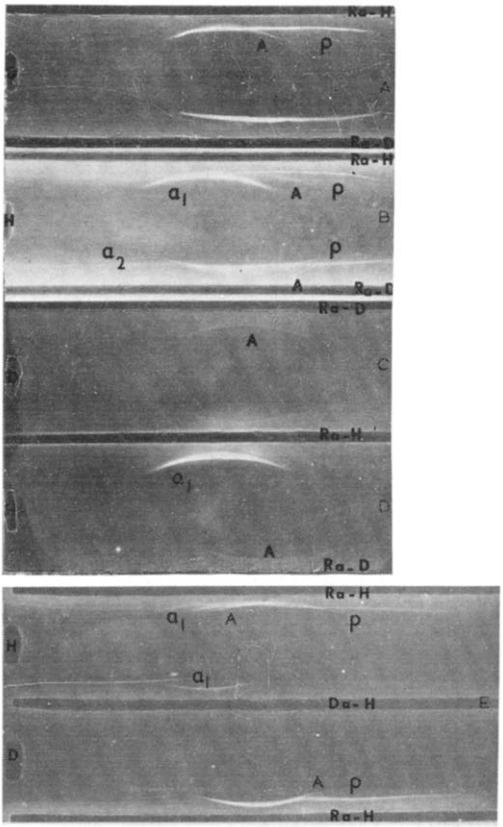

Fig. 4. Demonstration of a_1 esterase in horse serum. A and B. Slides revealed with β-naphthyl acetate. C and D. Duplicate slides revealed with α-naphthyl acetate. E. Slide revealed with β-naphthyl acetate. Da-H, donkey anti-horse antiserum. Other abbreviations as in Fig. 2.

Table I summarizes data on enzymatic and immunological differentiation of horse and donkey esterases.

TABLE I

Histochemical reaction	*Esterase-active lines detectable*					
	Horse				*Donkey*	
	a_1		α-ϱ		α-ϱ	
Antiserum:	*Ra-H**	*Ra-D***	*Ra-H*	*Ra-D*	*Ra-H*	*Ra-D*
α-Naphthyl acetate	+	−	−	−	−	−
β-Naphthyl acetate	+	−	+	+	+	+
Lipid Crimson	−	−	+	+	+	+

* Anti-horse rabbit antiserum.
** Anti-donkey rabbit antiserum.

Correlation of esterase components detected in immunoelectrophoresis and in starch gel electrophoresis

Compared to the previous results[8,10], analogous observations are obtained, but the electrophoretic location of esterases in starch and agar appears reverse. The identification was therefore attempted by eluting the active fractions from starch gel and analyzing them subsequently by immunoelectrophoresis.

Eluate of horse E_4 esterase. With β-naphthyl acetate the line of ϱ esterase was revealed, reacting with either anti-horse or anti-donkey antisera. This eluate contained nonesterase components, α globulins and a trace of albumin. The line of ϱ took the Lipid Crimson stain.

Eluate of horse E_5 esterase. With β- or α-naphthyl acetate, the arc of α_1-esterase was revealed, reacting with anti-horse antiserum. With both antisera, traces of ϱ-esterase were also seen using β-naphthyl acetate. Albumin was present in E_5 eluates in variable amounts.

Eluate of donkey E_4 esterase. Whatever portion of the active spot was cut off the starch gel layer, central (called E_4) or more anodic (called E_5), a single esterase-active line was detectable, corresponding to ϱ-esterase. This component reacts similarly with both antisera; it hydrolyzes β- but not α-naphthyl acetate or indoxyl acetate. The line is stained by Lipid Crimson.

Eluates of E_2 esterase of horse and donkey. These eluates, obtained in a similar way, from the same gel layer, yielded precipitation lines in β_1 zone, reacting with both antisera. No esterase activity, however, could be detected with any of the usual substrates.

Summing up, the eluates of horse and donkey esterase E_4 contain a lipoprotein esterase, reacting with anti-horse and anti-donkey antisera, migrating in agar faster than albumin. The eluate of horse esterase E_5 contains mainly a non-lipoprotein esterase, reacting with anti-horse antiserum, migrating in agar as α_1. Thus the relative migration rates are reversed in the two media: E_4, slower than E_5 in starch, becomes ϱ esterase in agar, definitely faster than α_1-esterase. In spite of this difference, due to the molecular sieve effect of starch gel, the identification of E_4 with ϱ-esterase and of E_5 with α_1-esterase can be established on the basis of their enzymatic and antigenic characters.

Immunological comparison of horse and donkey esterases

The esterase-containing eluates were tested in immunodiffusion; a convenient substrate was chosen for each antiserum. Thus, eluates of horse and donkey E_4 were compared using anti-donkey antiserum and revealed by β-naphthyl acetate; the esterase-active lines joined each other, demonstrating the antigenic identity of horse and donkey ϱ components (Fig. 5A).

Eluates of donkey and horse E_4 and E_5 were also compared using anti-horse antiserum and revealed by α-naphthyl acetate (Figs. 5B and 5C); only the α_1-esterase, contained in horse eluates, was revealed in such conditions.

Because of difficulties in observing a line ascribable to α_1-esterase in the immunoelectrophoretogram of whole horse serum, the absence in donkey serum of a hypothetical enzymatically inactive protein, antigenically related to horse α_1, could not be directly ascertained. Therefore, this absence was checked by absorbing, with rabbit anti-donkey antiserum, of all horse serum proteins that would precipitate with this

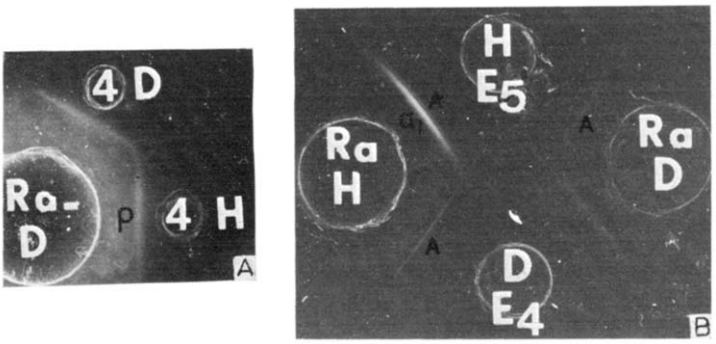

Fig. 5. Immunodiffusion tests of horse and donkey esterase -containing eluates of starch gel. A. Slide revealed with β-naphthyl acetate; 4 D and 4 H, respectively, eluates of donkey and horse esterases E_4. B. Slide revealed with α-naphthyl acetate; the albumin lines of horse and donkey coalesce for the two antisera. H E_5 and D E_4, respectively, eluates of horse E_5 and donkey E_4 esterases.

antiserum. This was carried out using (a) the whole anti-donkey antiserum: 7 ml were added to 0.25 ml of horse serum; (b) the globulin fraction prepared by $(NH_4)_2SO_4$ precipitation at 33% saturation: 4 ml of this fraction were added to 0.25 ml of horse serum. The supernatants obtained were tested in immunoelectrophoresis with rabbit and donkey anti-horse antisera. In both cases they yielded the α_1-esterase line; no other horse serum antigens were detectable in these supernatants.

DISCUSSION

The apparent discrepancy between previously reported antigenic identity of several analogous proteins in horse and donkey sera[2,7] and the presence of an esterase in horse serum and not in donkey serum[8,10], prompted an immunochemical study of esterases in the two species. It could be conceivable, indeed, to find in donkey serum an enzymatically inactive protein, antigenically related to horse esterase.

The demonstration of an esterase present in horse serum only, identifiable by its enzymatic properties, as free protein and as the enzyme–antibody complex, as well as by its antigenic properties, oppose this hypothesis.

Actually, not only the characteristic α_1 esterase activity was not found in donkey serum, but also cross-examination with anti-horse and anti-donkey antisera has shown that only the former contains corresponding antibodies. In fact, neither donkey serum reacting with anti-horse antiserum nor horse serum reacting with anti-donkey antiserum permits the demonstration of α_1-esterase. Moreover, the donkey immunized with horse serum formed antibodies against this component.

The second esterase investigated, E_4 or ϱ, was found present in horse and donkey sera and is detectable using both anti-horse and anti-donkey antisera. Moreover, the test by immunodiffusion demonstrated the antigenic identity of these proteins in two species.

Therefore, horse and donkey are equipped with a set of identical (or closely similar) components, typified by ϱ-esterase, defined by four parameters: activity, charge, lipid moiety and antigenic structure. On the other hand, during the phylo-

genetic evolution the horse has acquired* an unique esterase. Doubtlessly, this enzyme must have a physiological significance; yet despite its high activity and broad specificity, its role does not seem of primary metabolic importance, as other species of Equidae are not provided with it**.

Concerning the substrate specificity, the marked difference in behavior of ϱ-esterase towards α- and β-naphthyl acetates deserves mention. The classification of esterases being based on the length of the carboxylic acid chain and on the aliphatic or aromatic nature of the alcoholic compound involved in the ester used as substrate, it seemed insignificant to numerous investigators to use either α or β isomer. In a few instances where both were used, similar results have been reported[17,18]. The hydrolysis of the esters of α-naphthol by the human serum was reported as more efficient than that of β isomers[19]; this difference was not, however, ascribed to any particular enzyme or serum fraction.

Besides its biochemical significance proper, this difference in specificity was especially helpful in our study in permitting a direct immunological demonstration of α_1-esterase: if α-naphthyl acetate is used, only the α_1 active lines are revealed, whereas, with β-naphthyl acetate, the superimposition of α_1- and ϱ-esterase lines prevents the identification.

Similar specificity of a non-lipoprotein esterase, reacting intensely with α-naphthyl acetate, and of a lipoprotein esterase, hydrolyzing only the β isomer, was observed in sheep[20] and avian sera[14]. Such correlation might therefore be considered as a general phenomenon.

The association of lipid and esterase properties was reported in many animal species[11,13,14,20–24]. The metabolic significance of this fact remains still incompletely elucidated. Is the lipid moiety directly involved in the enzymatic activity? Are enzymes carriers of lipids or lipoproteins carriers of enzymes? Contradictory statements are found in the literature; thus, for one arylesterase of human liver, the function of lipid carrier was established[25], while the slow-migrating serum lipoproteins are assigned the role of esterase transport[26].

Finally, the fact that in the antigen–antibody complexes formed by the two esterases studied, not only the activity of the enzyme alone is preserved but also the requirement of a particular steric configuration of the substrate indicates that presence of antibody has no impeding effect on the substrate fixation.

CONCLUSION

The immunochemical results permitted the histochemical observation concerning the "horse-specific" esterase to be confirmed and extended.

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* Acquired rather than preserved an ancestral component, as is suggested by the results of the ontogenetic study of horse esterases[27].

** Donkey and Zebra[28].

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