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QUANTITATIVE DETERMINATION OF PROTEOLYTIC, LIPOLYTIC AND HAEMOLYTIC ACTIVITIES IN SEMEN OF SOME ANIMAL SPECIES

By

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Semen plasma contains several components which have enzymatic activity, and the number of enzymes which are described increases steadily.

T. Mann (1964) mentions in his book "Biochemistry of Semen and of the Male Reproductive Tract" a number of enzymes which are found in semen and semen plasma, such as hyaluronidase, transaminases, desaminases, phospholipases, egg yolk coagulating enzyme and phosphatases. Further, *Mann* describes the enzymes vesiculase, coagulase, fibrinolysin, fibrinogenase, aminopeptidase, pepsin and arginin-ester-hydrolyzing enzymes. All these are assigned by *Mann* to the proteolytic enzymes. Besides a haemolyzing factor is found in bull's semen.

In connection with artificial insemination problems have arisen due to the fact that enzymes of the semen have decomposed certain constituents of the diluent. This has led to physical changes in the diluted semen, and to the formation of spermicidal substances. This applies particularly to the semen from the goat (*Roy* 1957; *Iritani & Nishikawa* 1962, 1963; *Aamdal, Lyngset & Fossum* 1965).

Thus it appeared to be a suitable task to carry out comparative quantitative and qualitative investigations of different enzymatic activities in the semen of the domestic animals.

MATERIALS AND METHODS

Semen from the goat, ram, bull, boar and dog was examined with respect to proteolytic and lipolytic activity and to enzymes which cause change in an egg yolk substrate. This change resulted in increased opacity or to clarifying of the medium (lysis). The enzymatic factors, which were responsible for these changes, were called egg yolk turbidity factor (EYTF) and egg yolk lysis factor (EYLF). Some semen samples were examined with respect to haemolytic activity.

Semen of 10 different animals of the said species were examined quantitatively for proteolytic and lipolytic activity and egg yolk factors. In addition, similar investigations were carried out on the semen from a stallion.

In all the species except the dog the semen was collected by means of artificial vaginas. In the dog the semen was collected by digital manipulation of the penis. For the species which have a more restricted reproductive season the investigations were performed in this season.

The enzymatic investigations of the semen were carried out as soon as possible after collection (within 2 hours), in order to prevent any possible action of bacterial enzymes; besides the samples were kept at 4°C from the collection to the titration of the enzymes.

Demonstration and titration of enzymes. The principle of agar diffusion described by *Sandvik* (1962) for indication and quantitative demonstration of bacterial proteolytic enzymes was used for quantitative determination of proteolytic and lipolytic enzymes, the egg yolk factor and haemolytic activity.

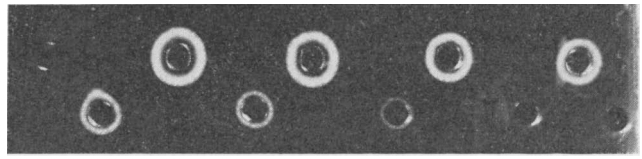
Proteolytic enzymes. For the demonstration and titration of proteolytic enzymes a method was used which has been described by *Sandvik* (1962). The substrate had the following composition:

Agar (Difco, Bacto-Agar, 0140-01)	1.40 %
Sodium caseinate (Eastman Kodak, P914), added as 4 % solution, pH 6.2	1.00 %
Merthiolate	0.01 %
$MgCl_2$ (added as 10 % solution)	0.004 M
Destilled water to	100 %
pH 6.2	

The agar was in the melted state and after the addition of the other ingredients it was poured on to glass plates having plastic frames, so that an even layer, 2 mm thick, was formed. In this agar, by means of a cork borer, wells were made 6 mm in diameter. The wells were

made at such distance from one another that there could be no interference between the zones. In the wells 0.025 ml of the enzymatic material or a dilution of this was placed with a micropipette. The casein plates were incubated at 37°C for 18 hours.

When proteolytic enzymes diffused out into the agar, an opaque zone was formed as a result of decomposition of the casein (Fig. 1). As the zone extended, there appeared at times a secondary lysis be-



F i g. 1. Precipitation zones in casein agar; 2-fold serial dilutions of dog semen. Diluent: Saline.

tween the well and the opaque zone. As in this procedure proteolytic activity is registered on the basis of the capacity to precipitate casein, these enzymes are called *"casein-precipitating enzymes"* or *"CP-enzymes"*.

In titrating the semen for enzyme activity a two-fold dilution in physiological saline was prepared. The maximum dilution, in which the enzyme activity was registered in 0.025 ml, was defined as a diffusion unit. This applies to all the enzymes investigated.

Lipolytic enzymes. For the demonstration and titration of lipolytic enzymes use was made of tributyrin as substrate. Tributyrin was stirred out in a basis-agar having the following composition:

Ordinary nutrient agar	1.4 %
Tributyrin (Mercks Reagenzien)	0.4 %
Tween 80	2 %
Merthiolate	0.01 %
Physiological saline (in which tributyrin and Tween 80 were mixed)	16 %
Destilled water to	100 %
pH 7.0	

The demonstration and titration of tributyrin-decomposing enzymes (tributyrinases) were performed in the same way as for the proteolytic enzymes. A clarification occurred here round the wells, as a sign of enzyme activity (Fig. 2). Reading was taken after 48 hours of incubation at 37°C.

Egg yolk factors. For the demonstration and titration of egg yolk factors use was made of a medium consisting of equal portions of a 3 % nutrient agar and a 10 % suspension of egg yolk in nutrients broth. The agar and the egg yolk suspension were mixed at 46°C.

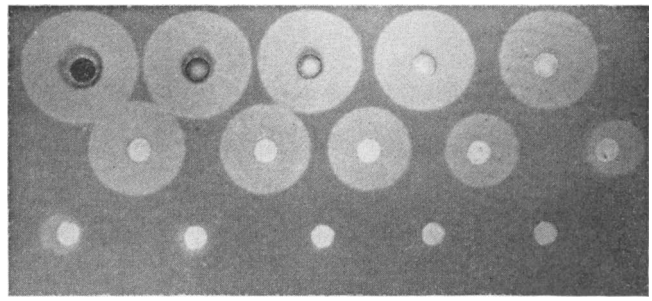


Fig. 2. Lysis zones in tributyrin agar; 2-fold serial dilutions of goat semen. Diluent: Saline.

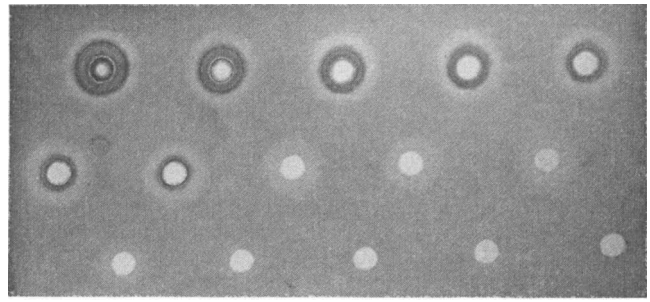


Fig. 3. Precipitation (inner) and lysis (outer) zones in egg yolk agar; 2-fold serial dilutions of goat semen. Diluent: Saline.

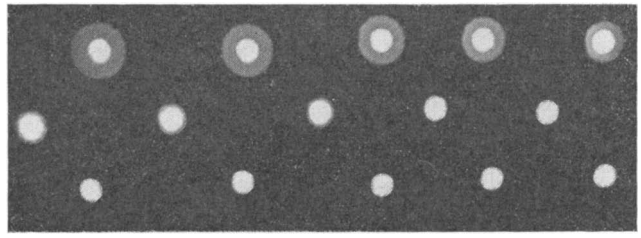


Fig. 4. Haemolyzed zones on blood agar; 2-fold serial dilutions of bull semen. Diluent: Saline.

Merthiolate (final concentration 0.01 %) was added, and the mixture was poured on to glass plates (*Fossum* 1963). The pH of the medium was 7.0. Dilution and titration were performed as described above, and the plates were incubated for 48 hours at 37°C before reading. Fig. 3 shows zones which appeared in a dilution series of goat semen on such a substrate.

Haemolysins. For the demonstration and titration of haemolytic activity use was made of the following composition of the agar (*Fossum* 1963):

Agar (Difco, Bacto-Agar, 0140-01)	1.4 %
NaCl to a final concentration of	0.95 %
Washed bovine erythrocytes	4 %
Merthiolate	0.01 %

pH 6.5

Dilution was effected as described above. The plates were incubated for 16 hours at 37°C before reading. Fig. 4 shows haemolyzed zones in a dilution series of semen on such a plate.

Counting of sperm. The number of sperm in the semen samples was counted according to the haemocytometer method with a Bürke-Turk haemocytometer and Hayem's solution as diluent except for boar semen where 2 % chloromidol was used as diluent. In goat, ram and bull the semen was diluted 1:200, in the other species 1:100.

RESULTS

The results of some of the enzymatic investigations will be seen from Table 1. Sperm numbers are also included.

The concentration of proteolytic (CP) enzymes in the samples of goat's semen varied from 64 to 1000 diffusion units per 0.025 ml. We disregard sample no. 8, in which there was only a trace of proteolytic enzymes and no spermatozoa were demonstrated, indicating that this sample was only secretion from the accessory sexual glands. Semen from the goat contained relatively high concentrations of tributyrinase and egg yolk factors. Semen from the goat produced a constant opaque zone between the well and an outer lysis zone. This egg yolk turbidity factor could be registered in dilutions up to 1:280, where lysis was registered in far higher dilutions.

Semen from the ram contained amounts of proteolytic enzymes similar to those in semen of the goat, but the variation from one animal to another was less (120—1000 diffusion units). In semen of the ram there was very little tributyrin-decomposing activity, but in egg yolk medium lysis occurred in dilutions up to 1:100—1:200. Opacity did not occur at all.

The semen of bulls showed little proteolytic activity and the concentrations of tributyrinase and "egg yolk lysis factor" were small. Nor in this case did any opaque zone appear in the egg yolk medium.

Table 1. Occurrences of enzymes in the semen from certain animal species, expressed as diffusion units per 0.025 ml.

Species	No.	CP-enzymes	Tributyrinase	EYLF	EYTF	No. of sperm/mm³
Goat	1	500	1000	4000	250	2 648 300
	2	1000	1000	2000	250	3 626 500
	3	1000	4000	4000	250	3 576 500
	4	250	500	1000	64	3 186 500
	5	250	500	1000	64	2 906 500
	6	250	1000	2000	128	2 566 500
	7	500	500	2000	32	2 495 000
	8	4	1000	4000	250	0
	9	64	250	500	16	1 880 000
	10	250	500	1000	32	2 431 650
Ram	1	1000	8	250	0	
	2	1000	8	128	0	
	3	1000	8	128	0	
	4	250	8	32	0	
	5	500	8	32	0	
	6	250	8	128	0	3 995 000
	7	250	8	128	0	
	8	250	16	128	0	
	9	250	16	128	0	
	10	128	16	64	0	
Bull	1	250	250	32	0	1 556 000
	2	16	250	32	0	907 500
	3	32	250	32	0	812 500
	4	32	250	32	0	1 262 500
	5	32	128	64	0	1 030 000
	6	128	128	64	0	1 130 000
	7	128	64	32	0	1 065 000
	8	64	128	250	0	1 250 000
	9	64	250	64	0	1 055 000
	10	64	250	128	0	
Boar	1	0	4	0	0	168 250
	2	4	4	4	2	282 500
	3	8	16	4	2	282 500
	4	8	8	2	1	285 000
	5	0	16	8	4	341 500
	6	2	8	4	2	156 500
	7	2	8	8	4	233 000
	8	0	16	8	4	114 000
	9	0	16	8	4	279 000
	10	0	16	16	8	241 650

Table 1. Continued.

Species	No.	CP-enzymes	Tributy-rinase	EYLF	EKTF	No. of sperm/mm³
Dog	1	128	8	4	0	
	2	128	64	32	0	228 250
	3	500	2	8	0	
	4	128	128	16	0	240 250
	5	500	128	16	0	320 750
	6	250	16	0	0	
	7	500	16	0	0	
	8	128	64	16	0	
	9	128	4	0	0	
	10	250	32	0	0	

In boar's semen only small quantities of enzymes were found. In some cases no activity could be demonstrated at all. In the egg yolk medium it was possible, however, regularly to register an opaque zone similar to that found for goat's semen. The causal factor, however, was only found in extremely small quantities. Dog's semen contained relatively large quantities of proteolytic enzymes, whilst it showed little activity in relation to tributyrin and egg yolk.

In the one sample of stallion semen no proteolytic activity was found, relatively scanty amounts of tributyrinase, but large quantities of the "egg yolk lysis factor".

From each of the investigated animal species at least one semen specimen was examined for haemolytic activity. In the case of the bull, semen from 17 different animals was examined. The results appear from Table 2.

Table 2. Occurrence of haemolysins in semen; diffusion units per 0.025 ml.

Species	Number of animals	Haemolysins
Goat	3	0
	1	250
Ram	5	0
Bull	17	125—250
Boar	3	0
	1	4
Dog	2	0
Horse	1	32

In goat semen we found relatively large amounts of haemolysin in one of the four specimens examined, whilst the other three were negative. The specimens from ram and dog were also negative, whilst large amounts of haemolysins were regularly found in bull semen. Boar semen was negative or contained only traces of haemolysins, whilst the single specimen from the horse showed some haemolytic activity.

DISCUSSION

As mentioned in the description of method, in these investigations sodium caseinate has been used as substrate in the demonstration of proteolytic enzymes. Thus the active factors may be called "casein precipitating enzymes" (*Sandvik* 1962). Unreservedly we cannot put a sign of equation between these activities and any of the activities which *Mann* ascribes to the proteolytic enzymes, such as vesiculase, coagulase, fibrinolysin, fibrinogenase, aminopeptidase, pepsin and arginin-ester-hydrolyzing enzymes.

The factor which *Iritani & Nishikawa* (1963) and *Mann* (1964) call the egg yolk coagulating factor in semen appears to be identical with that which in our investigations is termed "egg yolk turbidity factor". This term is used because it is in agreement with the one used for the corresponding bacterial enzymes.

There appear to be two different enzymes which are responsible for the increased opacity and lysis. Whilst the former activity is thought to be due to a lecithinase, lysis seems to be due to a fat-decomposing enzyme. There seems to be a certain relation between the amount of tributyrinase and egg yolk factor.

Haemolytic activity in the semen from the bull is determined by *Romaniuk* (1961) and *Molinary* (1964). Whereas we found relatively large concentrations of haemolysins in the semen from all the bulls examined, *Romaniuk* found haemolytic activity in only 99 out of 277 ejaculations.

The agar diffusion method is used with good result for corresponding bacterial enzymes. The method is quick and accurate and is in some respects more sensitive than the methods used previously. It seems to be well fitted for the titration of such enzymes in semen.

These investigations show that the concentrations of the different enzymes in the semen from various animal species vary

to a great extent. Within the same species there is some variation from one animal to another and also among specimens taken at different times from the same animal, but in general the level is the same.

The significance of many of the enzymes of semen is at present obscure. Hyaluronidase, for example, has been considered important for the fertilization (*Mann* 1964). In connection with artificial insemination it has been found that semen contains enzymes which decompose certain of the substances in diluents (*Aamdal, Lyngset & Fossum* 1965). In this way various enzymatic activities in semen have become very important in practical breeding work. This applies in particular to the goat when diluents with egg yolk are used. Of the species investigated the goat is the only one the semen of which contains large amounts of the egg yolk turbidity factor. Likewise the special circumstances connected with dilution are only recorded in the dilution of goat semen. This is in agreement with the investigations by which a deleterious effect of egg yolk in diluent for goat semen was demonstrated (*Aamdal, Lyngset & Fossum* 1965).

Boar semen also contains traces of a similar enzyme. Egg yolk is used as a component of diluents for boar semen, but at present this enzyme activity is not put into connection with storage problems. To which extent the other enzyme activities investigated are of importance for the viability of stored semen cannot be stated at present; further investigations are needed before the significance of these enzymes can be shown.

The present investigations indicate that variations of the enzyme concentrations in semen are due more to differences between species than between individuals within the same species. Thus a regular control of semen does not seem to have any great significance.

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SUMMARY

1. Methods for quantitative determination of proteolytic, lipolytic and haemolytic activities and egg yolk factors in semen are described.
2. Comparative quantitative determinations of these enzyme activities in the semen of goat, ram, bull, boar, dog and horse have been carried out.
3. Special interest attaches to the egg yolk turbidity factor which is found in goat semen and, in small concentrations, also in boar semen.
4. As each species appears to have its specific enzyme pattern, regular enzymatic control does not seem to have great significance.

ZUSAMMENFASSUNG

Die quantitative Bestimmung der proteolytischen, lipolytischen und hämolytischen Aktivität in Samen von verschiedenen Tierarten.

1. Methoden zur quantitativen Bestimmung der proteolytischen, lipolytischen und hämolytischen Aktivität sowie „egg yolk factors" in Samen werden beschrieben.
2. Vergleichende quantitative Bestimmungen der erwähnten Enzymaktivitäten in Samen von Ziegenbock, Widder, Stier, Eber, Hund und Hengst sind vorgenommen worden.
3. Besonderes Interesse ist mit dem „egg yolk turbidity factor" verbunden. Dieser kommt in Samen vom Ziegenbock und in kleineren Konzentrationen in Samen vom Eber vor.
4. Weil jede Tierart anscheinend ihr eigenes spezielles Enzymmuster besitzt, scheint eine routinemässige enzymatische Kontrolle von Samen nicht von grösserer Bedeutung zu sein.

SAMMENDRAG

Kvantitativ bestemmelse av proteolytisk, lipolytisk, og hemolytisk aktivitet i sæd fra noen dyrearter.

1. Det er beskrevet metoder til kvantitativ bestemmelse av proteolytiske, lipolytiske og hemolytiske aktiviteter samt „egg yolk factors" i sæd.

2. Det er foretatt sammenlignende kvantitativ bestemmelse av de nevnte enzymaktiviteter i sæd av geitebukk, vær, okse, råne, hund og hingst.
3. Spesiell interesse knytter seg til "egg yolk turbidity factor", som forekommer i sæd av geitebukk og i små konsentrasjoner også i sæd fra råne.
4. Da hver dyreart synes ha sitt spesielle enzymmønster synes rutinemessig enzymatisk kontroll av sæd ikke å være av vesentlig betydning.

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