Acta vet. scand. 1971, *12*, 417—428.

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PROTEOLYTIC ENZYMES AND BIOLOGICAL INHIBITORS

V. SEROLOGICAL RELATIONSHIP BETWEEN BOVINE AND SWINE TRYPSINS AND BOVINE α-CHYMOTRYPSIN

By
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Bovine and swine trypsins have been reported to be similar in various respects, such as size, charge, terminal amino acids, and the amino acid sequence in the active centre of the molecules, although there are differences in the primary structures (*Travis & Liener* 1965 a, b). The 2 trypsins are, in most cases, equally affected by the naturally occurring trypsin inhibitors as tested by the combination of electrophoresis of the inhibitor-containing materials and the casein precipitating inhibition test (electrophoretic CPI-test) (*Fossum* 1970 a, b, c, d). Chymotrypsin, which is known to have many regions in the molecule identical to those of trypsin (*Walsh & Neurath* 1964), is, in many cases, also affected by the same inhibitors as trypsin (*Vogel et al.* 1968, *Fossum* 1970 a, b, c, d).

Sandvik (1962) used the electrophoretic CPI-test in serological studies of bacterial proteinases in order to distinguish between the naturally occurring inhibitors in the α- and β-globulin fractions, and the antiproteinases in the γ-globulin fractions of immune rabbit sera. It was found that proteinases, from different species of bacteria, were usually serologically different. The serological differentiation of the extracellular proteinases of certain groups of microorganisms has later been used for classification purposes (*Sandvik & Fossum* 1965, *Sandvik* 1967, 1969, *Sandvik & Hagen* 1968, *Rutqvist* 1969).

The aim of the present work was to determine the serological relationship between bovine and swine trypsins and bovine α-chymotrypsin. The electrophoretic CPI-test and the Kunitz method (*Kunitz* 1947) were used for the estimation of the enzyme inhibitory capacity of the antienzymes, and the double diffusion technique (*Crowle* 1961, *Ouchterlony* 1968) was used in precipitation experiments.

MATERIALS AND METHODS

Enzymes. Trypsin from bovine pancreas (type III, 2 × crystallized, lot 97B-8000), and α-chymotrypsin from bovine pancreas (type II, 2 × crystallized, lot 86B-0470) were obtained from Sigma*); acetyltrypsin from bovine pancreas (batch no. 24859) and trypsin from swine pancreas (crystallized, batch no. 3647) were obtained from Koch-Light**).

Sera. Antiproteinases against bovine and swine trypsins and α-chymotrypsin were produced in rabbits by injecting the enzymes (1 mg per ml) mixed with equal amounts of Freund's complete adjuvant (Difco). The antigens were injected subcutaneously into rabbits in amounts of 1.0, 2.0, and 4.0 ml at 6 days' intervals. One week after the 3rd injection the rabbit sera were tested for the presence of antienzymes. Two additional injections of 0.5 and 1.0 mg of the enzymes were then given intravenously on the 20th and 32nd day of immunization, and the sera were tested 1 week after each injection. The rabbits were finally bled on the 42nd day after the first injection.

The immunoglobulins were isolated by fractionation with sodium sulphate as described by *Stelos* (1967).

Determination of antienzymes. Separation of the naturally occurring inhibitors and the antienzymes, and the demonstration of the inhibitory activity of the antienzymes, were performed as described by *Sandvik* (1962). Samples of 8—12 µl of serum were applied onto paper (Schleicher and Schüll filter paper, no. 2043 bmgl.) and electrophorized at 120 v for 18 hrs. in a 0.05 M phosphate buffer, pH 6.2, using LKB***) electrophoresis apparatus, type 3276 BN. The paper strips were then transferred to the surface of a caseinate-containing agar. After incubation at 37°C for 2 to 3 hrs. the strips were removed from the medium and replaced by narrow (0.4 mm) strips of filter paper moistened with the enzyme-containing solution (0.002—0.01 mg enzyme per ml). Precipitation occurred along the enzyme-containing strips which were removed after 15—18 hrs.' incubation at 37°C. Inhibition was indicated by interruptions of the white precipitation

*) Sigma Chemical Company, St. Louis, USA.

**) Koch-Light Laboratories Ltd., Colnbrook, Buckinghamshire, England.

***) LKB-produkter, P.O. Box 12035, Stockholm 12, Sweden.

zones, or by a more or less narrowing of these zones, depending upon the inhibitory activity. The naturally occurring inhibitors in rabbit sera were localized on the anode side of the line of application (Figs. 1, 2, and 3) while the inhibition due to antibodies was situated at, or near, the line of application under the present conditions. The sera, or the isolated immunoglobulins, were, in some cases, diluted with saline before the electrophoresis was carried out.

The Kunitz method (*Kunitz* 1947) was used for the determination of the inhibitory activity of whole fresh sera, and of the fractionated immunoglobulins according to the modification of *Fossum* (1970 a).

The precipitating interaction of the enzymes and the antisera, or the fractionated immunoglobulins, was tested by the double diffusion technique of Ouchterlony (*Crowle* 1961, *Ouchterlony* 1968) by placing different dilutions of the antisera in a central well, and different dilutions of the enzymes in surrounding wells.

RESULTS

The effect of the 3 antisera on the various enzymes at different stages in the immunization process is shown in Table 1. It can be seen that after 18 days of immunization the enzymes are only inhibited by the homologous antisera. After further 12 and 24 days of immunization, the anti-bovine trypsin also inhibits swine trypsin and α-chymotrypsin, and the anti-swine trypsin inhibits bovine trypsin but not α-chymotrypsin. Anti-α-chymotrypsin does not inhibit either bovine or swine trypsins.

Table 1. The inhibitory effect of antienzymes for bovine and swine trypsins and α-chymotrypsin upon the 3 enzymes at different stages in the immunization process, based on the electrophoretic CPI-test.

Antienzymes	Days of immunization	Enzymes		
		bovine trypsin	swine trypsin	α-chymotrypsin
Anti-bovine trypsin	18	+++*)	—**)	—
	30	++++	+	+
	42	++++	++	++
Anti-swine trypsin	18	—	+++	—
	30	+	++++	—
	42	++	++++	—
Antiα-chymotrypsin	18	—	—	+++
	30	—	—	++++
	42	—	—	++++

*) ++++, +++, ++, +: various degrees of inhibition.
**) —: no inhibition observed.

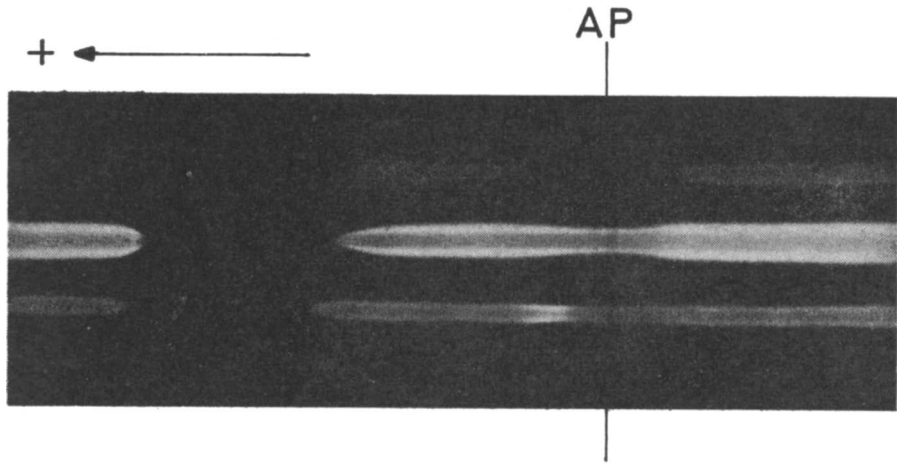

Figure 1. Electrophoretic CPI-test of anti-bovine trypsin. The enzymes used are (downwards): Bovine trypsin (0.01 mg per ml), swine trypsin (0.005 mg per ml), and α-chymotrypsin (0.005 mg per ml). The electrophoresis was carried out in 0.05 M phosphate buffer at pH 6.2 for 18 hrs. The zones of inhibition near the line of application (AP) are due to antienzymes in the γ-globulin fractions, while the zones of inhibition on the anode side are due to the naturally occurring inhibitors in rabbit serum.

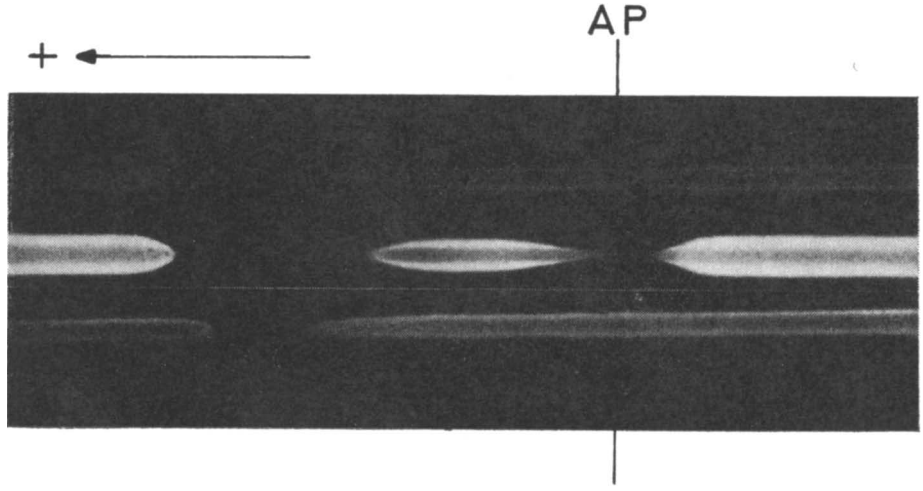

Figure 2. Electrophoretic CPI-test of anti-swine trypsin. The enzymes used, and other conditions, are the same as in Fig. 1.

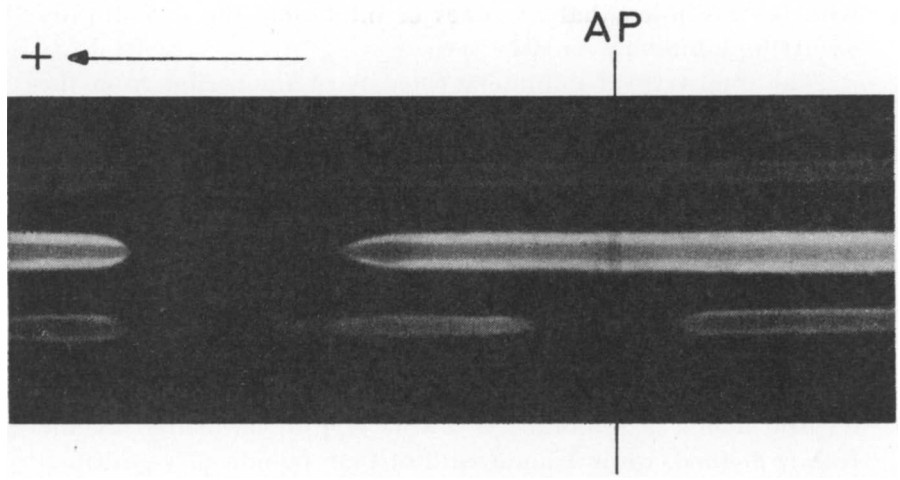

Figure 3. Electrophoretic CPI-test of antiα-chymotrypsin. The enzymes used, and other conditions, are the same as in Fig. 1.

The effect of the antienzymes from the 42nd day of immunization is shown in Figs. 1, 2, and 3. Acetyltrypsin (bovine) was inhibited in the same manner as bovine trypsin. Table 2 shows the highest dilutions of each of the 3 antisera resulting in inhibition of the enzymes in question. While the 3 sera inhibit the homologous enzymes to approximately the same extent (1:50, 1:40, and 1:40 for the bovine and swine trypsins, and α-chymotrypsin, respectively) the heterologous inhibition is about 1 tenth of the homologous inhibition (Table 2).

The electrophoretic CPI-test performed on the isolated γ-globulins gave results similar to those obtained with the whole sera,

Table 2. Highest dilution of antienzymes (after 42 days of immunization) for bovine and swine trypsins and α-chymotrypsin resulting in inhibition of the 3 enzymes, based on the electrophoretic CPI-test.

Antienzymes	Enzymes		
	bovine trypsin	swine trypsin	α-chymotrypsin
Anti-bovine trypsin	1:50*)	1:6	1:6
Anti-swine trypsin	1:5	1:40	0**)
Antiα-chymotrypsin	0	0	1:40

*) the sera were electrophorized in the dilutions 1:1, 1:2, 1:4, 1:5, 1:6, 1:8, 1:10, 1:20, 1:40, 1:50, and 1:60.

**) 0: no inhibition observed.

with the exception that no zones of inhibition due to naturally occurring inhibitors could be seen.

The total trypsin inhibitory capacity of the serum from the rabbit immunized with bovine trypsin increased by 50 % (from 0.8 mg to 1.2 mg of bovine trypsin inhibited by 1 ml of the serum) from the beginning to the end of the immunization period (homologous system) as tested by the Kunitz method. The Kunitz test, performed on the isolated γ-globulins, gave results similar to those obtained with the electrophoretic CPI-test shown in Table 2, although greater amounts of enzymes and γ-globulins had to be used in the first method. Thus, 0.1 ml (3 mg) of γ-globulins of anti-bovine trypsin caused 63 % inhibition of bovine trypsin, and 7 % inhibition of swine trypsin, measured by the Kunitz method, while 1 hundredth of that amount of γ-globulins resulted in distinct zones of inhibition of both bovine and swine trypsin in the electrophoretic CPI-test.

The results of the precipitation experiments carried out on the homologous and heterologous systems are shown in Table 3. For the heterologous systems, precipitation is seen only with anti-bovine trypsin and swine trypsin. To obtain this cross precipitation, larger amount of enzyme and lower dilutions of the heterologous serum had to be used than that necessary in the homologous systems. While precipitation occurred in the homologous systems 18 days after immunization, the precipitation in the anti-bovine trypsin and swine trypsin system was first seen after 30 days.

The double diffusion of bovine and swine trypsins, acetyltrypsin, and α-chymotrypsin against anti-bovine trypsin is pre-

Table 3. Precipitation obtained by the double diffusion technique by using different dilutions of the antisera and different amounts of the enzymes.

	Enzymes		
Antienzymes	bovine trypsin	swine trypsin	α-chymotrypsin
Anti-bovine trypsin	+ (0.05, 1:10) *)	+ (0.1, 1:2)	0**)
Anti-swine trypsin	0	+ (0.01, 1:10)	0
Antiα-chymotrypsin	0	0	+ (0.01, 1:10)

*) lowest amount of enzyme (mg per ml), and highest dilution of serum resulting in precipitation.

**) 0: no precipitation observed.

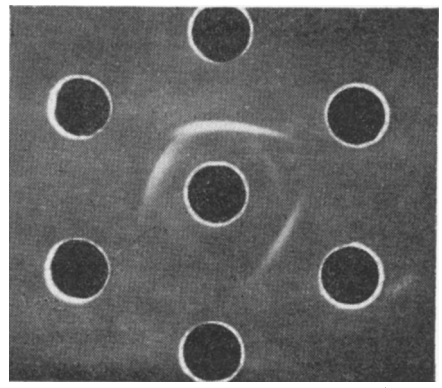

Figure 4. Double diffusion test with anti-bovine trypsin (central well), and bovine trypsin (1 mg per ml), swine trypsin (0.1 mg per ml), bovine trypsin (0.1 mg per ml), bovine trypsin (0.01 mg per ml), α-chymotrypsin (1.0 mg per ml), and acetyltrypsin (bovine, 1 mg per ml) clockwise from the top.

sented in Fig. 4. All these enzymes, except α-chymotrypsin, react with anti-bovine trypsin, but spurs are formed both between bovine and swine trypsins, and bovine trypsin and acetyltrypsin.

DISCUSSION

The results of the electrophoretic CPI-test showed that anti-bovine trypsin also inhibited swine trypsin and α-chymotrypsin, and that anti-swine trypsin inhibited bovine trypsin. By the double diffusion technique, cross precipitation was only demonstrated between anti-bovine trypsin and swine trypsin. No interaction was observed between anti-swine trypsin and α-chymotrypsin, or between antiα-chymotrypsin and bovine or swine trypsins by any of the methods used. The results obtained by the double diffusion technique are in accordance with those of *Arnon & Schechter* (1966) and *Arnon* (1967), who found that γ-globulins against bovine trypsin inhibited the chymotryptic (bovine) digestion of casein to some extent, and that chymotrypsin could cause partial inhibition of precipitation of bovine trypsin-antitrypsin. They found also an almost total absence of precipitation between chymotrypsin and antitrypsin. The present results are also in accordance with the results of *Pfleiderer et al.* (1970) who found no precipitation in the anti-bovine trypsin-bovine chymotrypsin or antichymotrypsin-trypsin systems. They found, how-

ever, weak immunological similarities between bovine chymotrypsin and bovine trypsin by use of the Osserman technique (*Osserman* 1960), in which chymotrypsin was found to cause a deviation in the precipitation line of bovine trypsin-anti-bovine trypsin. *Pfleiderer et al.* also found that anti-bovine trypsin precipitated swine trypsin with a spur formation in a homologous system similar to that shown in Fig. 4. In the heterologous systems in which inhibition occurred, this was, in the present work, about 10 % of that in the homologous systems (Table 2). *Pfleiderer et al.* found that the reactivity of swine trypsin with bovine antitrypsin varied, according to the different methods used, between 10 and 1 % of that of the homologous system, while bovine chymotrypsin showed only a slightly detectable interaction with anti-bovine trypsin. The stronger interaction between bovine chymotrypsin and anti-bovine trypsin reported by *Arnon & Schechter* is more in accordance with the results obtained in the present work. The discrepancy of the results may be due to the principle of the tests used. Thus, in the case of the electrophoretic CPI-test, as well as the work of *Arnon & Schechter,* the tests are based upon the inhibition of the functional activities of the enzymes. Most enzyme antibodies have an inhibitory effect upon the corresponding enzyme (*Cinader* 1967, *Cinader & Lepow* 1967, *Pollock et al.* 1967), especially when using substrates of high molecular weights. When synthetic substrates, such a benzoyl-arginine-p-nitroanilide are used, a much smaller inhibitory effect of antitrypsin was observed than when casein was used (*Arnon*). Although no comparison has been performed between the electrophoretic CPI-test and the hemagglutination inhibition test and the Osserman technique used by *Pfleiderer et al.* the high level of agreement between the results obtained by them and those presented here, indicate that the electrophoretic CPI-test is highly sensitive.

The length of the immunization period, the immunization routes, and the amount of enzymes used for immunization, should also be emphasized. In the present investigation the immunization period included a total of 42 days and the total amount of enzymes given to each animal was 5.5 mg, given by 3 subcutaneous and 2 intravenous injections. *Arnon & Schechter* used a corresponding immunization period, but 15 mg of the enzymes was given intramuscularly 3 times, while *Pfleiderer et al.* gave a total of 60 mg of the enzymes (bovine trypsin or chymotrypsin),

alternatively subcutaneously and intramuscularly, 8 times over a period of 4 months. The tendency for cross reactions has been found to increase during the immunization period (*Brown et al.* 1960, *Cinader*), a fact which is also in accordance with the present findings, in which cross reactions were not observed, in any case, until after 18 days of immunization.

The interaction between anti-swine trypsin and bovine trypsin does not seem to have been reported previously. *Ten Broeck* (1934) on the contrary, found, by anaphylactic experiments (Dale technique) on sensitized guinea-pigs, that the bovine and swine trypsins, and bovine chymotrypsin and chymotrypsinogen, did not interfere with each other.

The inhibitory activity of anti-bovine serum increased about 50 % from the beginning to the end of the immunization period in the present case. *Arnon* (1967) found that, when the inhibitory activity of immune sera was compared with that of normal sera, only very slight differences could be detected. *Hiramatu* (1941), on the contrary, found increased antitrypsin activity in immunized rabbit sera. It must, however, be emphasized in this connection, that the activity of the naturally occurring inhibitors is rapidly reduced by storage, so that after a few days the total inhibitory activity of the immune sera may even be less than that of normal fresh sera.

The spur formation in the case of acetylated bovine trypsin and bovine trypsin tested with anti-bovine trypsin in the double diffusion test (Fig. 4) indicates that the changes in the molecule resulted in minor changes in its antigenicity. The acetylation seems, however, to have little, if any, influence upon the ability of the enzyme to cross react with anti-bovine and anti-swine trypsins, based upon the inhibition test. The reactions of bovine acetyltrypsin with antibodies against native bovine trypsin does not seem to have been reported previously. The findings are different from the results reported by *Van Vunakis & Levine* (1963) on acetylated pepsinogen, which was found not to react with antipepsinogen.

In comparison with the serological relationship between bovine and swine trypsins reported above, anti-swine pepsinogen was found by *Van Vunakis & Levine* to cross precipitate horse and human pepsinogen. The nature of the spurring indicated antigenic differences between the 3 zymogens in these cases also.

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SUMMARY

The serological relationship between bovine and swine trypsins, and bovine α-chymotrypsin has been studied with rabbit antisera at different stages in the immunization period. By using paper electro-

phoresis to distinguish between the naturally occurring inhibitors and the antienzymes in the γ-globulin fractions, combined with the casein precipitating inhibition test (electrophoretic CPI-test) it was found that at 18 days after immunization the antienzymes inhibited only the homologous enzymes. After an additional 12 and 24 days the anti-bovine trypsin also inhibited swine trypsin and α-chymotrypsin, and anti-swine trypsin inhibited bovine trypsin, while antiα-chymotrypsin inhibited only the homologous enzyme. The enzyme inhibition in the heterologous systems was about $^1/_{10}$ of that in the homologous systems. Similar results were obtained by applying the Kunitz test to isolated γ-globulins. The total trypsin inhibitory activity of the whole anti-bovine trypsin serum increased 50 % from the beginning to the end of the immunization period (tested on bovine trypsin).

Using the double diffusion technique, cross precipitation only occurred between anti-bovine trypsin and swine trypsin.

Acetyltrypsin (bovine) was affected by the 3 antisera in a way similar to native bovine trypsin.

The results are discussed in relation to other reports concerning the serological relationship of animal proteinases.

SAMMENDRAG

Proteolytiske enzymer og biologiske inhibitorer.
V. Serologisk slektskap mellom bovint trypsin og svinetrypsin og bovint α-chymotrypsin.

En har undersøkt det serologiske slektskap mellom bovint trypsin og svinetrypsin og bovint α-chymotrypsin ved forskjellige trinn i immuniseringsprosessen ved å bruke antisera fremstilt på kaniner. Ved å benytte papirelektroforese for å skille mellom de naturlige forekommende inhibitorer og antienzymene, sammen med kaseinpresipitasjon-hemmingsreaksjon (CPI-test) fant en at enzymene ble hemmet bare av de homologe antienzymer etter en immuniseringstid på 18 dager. Etter ytterligere 12 og 24 døgn hemmet anti-bovint trypsin også svine-trypsin og α-chymotrypsin, og anti-svinetrypsin hemmet bovint trypsin. Antiα-chymotrypsin hemmet bare det homologe enzym. Hemmingen av aktiviteten i de heterologe enzymsystemer var ca. tiendeparten av hemmingen i de homologe systemer. Ved å benytte Kunitz reaksjon fikk en tilsvarende resultater ved bruk av isolerte γ-globuliner. Den totale hemming av antiserum mot bovint trypsin økte med 50 % fra begynnelsen til slutten av immuniseringsprocessen. Ved agar-geldiffusjon fikk en kryssspresipitasjon bare mellom anti-bovint trypsin og svinetrypsin. Acetyltrypsin (bovint) reagerte på tilsvarende måte som ordinært bovint trypsin med alle tre antisera.

En har sammenlignet resultatene med andre arbeider som angår serologisk slektskap mellom animalske proteolytiske enzymer.

(Received August 31, 1970).