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From the Department of Pharmacology and Toxicology, College of Veterinary Medicine, Helsinki, Finland.

CHARACTERIZATION OF PENICILLINASE INHIBITOR IN BOVINE SERUM

By

T. Honkanen-Buzalski

HONKANEN-BUZALSKI, T.: *Characterization of penicillinase inhibitor in bovine serum.* Acta vet. scand. 1982, *23*, 30—38. — Bovine serum containing penicillinase inhibitor activity was fractioned by gel filtration chromatography and ion exchange chromatography. The penicillinase inhibitor was principally located in the IgG-fraction and therefore appears to be an antibody.

When serum antipenicillinase activity was screened in material consisting of 54 animals, mastitis cows showed higher incidence of penicillinase inhibitor than healthy animals.

Serum penicillinase inhibitor had a slight effect on the MIC-value of penicillin against penicillinase producing staphylococci which indicates that this inhibitor could have some clinical influence in increasing bacterial sensitivity to penicillin.

penicillinase inhibitor; antipenicillinase; β-lactamase; bovine mastitis; S. aureus.

Penicillin resistance of Staphylococcus aureus depends on the production of β-lactamase (penicillinase). Four minor variants of the penicillinases have been described among various species of S. aureus (*Richmond* 1965 a, *Rosdahl* 1973). There have been speculation that during chronic infections natural antisera being raised to staphylococcal exocellular penicillinase may help to prevent the destruction of β-lactamase labile antibiotics under certain circumstances (*Cole* 1979). Also some in vivo experiments indicate that a smaller penicillin concentration is required to clear an infection if the animal is immunised by penicillinase (*Tacking* 1955). *Richmond* (1975) analysed the effects of specific β-lactamase antisera on penicillinase and reported both inhibitory and stimulatory effects. *Lie & Solbu* (1980) described penicillinase-inhibiting effects of bovine sera, but did not characterize the inhibitor molecules, however, their observations indi-

cate that the inhibition could be immunologically mediated: a significantly higher prevalence of penicillinase inhibitor was seen in mastitic cows than in healthy cows and a foregoing penicillin treatment of mastitis was seen to increase the serum antipenicillinase activity.

MATERIALS AND METHODS

Source of material

Serum samples from 24 mastitic cows, 10 healthy cows, 10 heifers and 10 calves were collected. The milk samples were analysed by conventional bacteriology.

Udder pathogenic strains of S. aureus (61 isolates) were collected from the daily material at the Mastitis Laboratory at the State Veterinary Medical Institute, Finland, and their penicillin sensitivity was tested by the agar diffusion method of *Casals & Pedersen* (1972). According to this method, 8 strains were completely resistant to penicillin (13 %) and these strains were used for production of penicillinase.

Production of penicillinase

The penicillin resistant strains of S. aureus ($n = 8$) were allowed to grow for 24 h at 37°C on Penassay Broth (Orion Diagnostica, Finland) containing 10 I.U. benzylpenicillin (Novocillin®, Novo Industri A/S, Denmark) per ml of broth. The broth cultures were filtrated through a Millipore filter with a pore size of 0.22 μm. The complete degradation of penicillin in these culture filtrates was tested by the lack of growth inhibition effect on the penicillin sensitive Bacillus stearothermophilus var. calidolactis. The penicillinase activity in these culture filtrates was tested by an agar diffusion method. Equal volumes of penicillin (0.1 I.U./ml) and staphylococcal culture filtrate were mixed and allowed to incubate for 1 h at 37°C. This mixture (50 μl) was then transferred into wells of 6 mm-diam. on an agar layer (2 mm) containing penicillin sensitive bacteria (B. stearothermophilus var. calidolactis) and incubated at 55°C for 3 h. The filtrate giving maximum penicillin inhibition (smallest growth-inhibition zone) was selected for further studies.

Determination of penicillinase inhibition by sera and serum fractions

The penicillinase inhibitor activity of sera and serum fractions were tested essentially as described by *Lie & Solbu* (1980). In this method penicillin and penicillinase are mixed together and the effect of serum addition was observed by analysing the remaining penicillin activity by agar diffusion on plates containing penicillin sensitive bacteria.

The serum or serum fractions (100 µl) and the penicillinase (100 µl) were mixed together and incubated for 1 h at 37°C then 100 µl of benzyl-penicillin, 0.1 I.U. per ml, was added and incubated another 1 h at 37°C. The penicillin concentration was adjusted as the maximum content of penicillin which was completely inactivated by penicillinase (final average penicillin concentration 0.033 I.U. per ml).

Penassay Seed Agar (Orion Diagnostica, Finland) plates seeded with B. stearothermophilus var. calidolactis were prepared to form a 2 mm thick layer on glass. Wells of 6 mm-diam. were cut and 50 µl of sample was transferred into them. Each determination consisted of a) sample, b) penicillin, c) sample + penicillin, d) sample + penicillinase, e) penicillin + penicillinase and f) sample + penicillinase + penicillin. Each mixture (a—f) was diluted with Penassay Broth to the same final volume (300 µl) before being transferred onto the plates. The plates were incubated at room temperature in a humid chamber for 1 h and then the temperature was raised to 55°C for 3 h. The growth inhibition zone around the wells was measured. The test was deemed positive (penicillinase inhibitor present) if the size of the penicillin growth inhibition zone in f) exceeded that of e).

Characterization of serum penicillinase inhibitor

a) Gel filtration chromatography. Plasma samples (3 ml) were subjected to gel filtration chromatography on Sephacryl S-300 (Pharmacia Fine Chemicals AB, Uppsala, Sweden) column size 2.6×94 cm (flow-rate 15 ml/h) eluted by 0.9 % NaCl at +4°C, 5 ml fractions were collected. The penicillinase inhibitor activity of each fraction was determined. For internal molecular weight standardisation, the elution pattern of BSA, IgG_1, IgG_2 and IgM was determined by analysing the fractions by double immunodiffusion against specific antisera (Miles Laboratories Inc., Elkhart, Indiana, USA). The location of fi-

brinogen in the plasma eluates was determined by mixing 10 NIH units of thrombin (Topostasin®, Hoffman-La Roche & Co AG, Basel, Switzerland) with 0.5 ml of the fractions, followed by observation for any coagulation.

b) Ion exchange chromatography. Serum samples (9 ml) were dialyzed against 0.0175 mol/l phosphate buffer, pH 6.8 and subjected to ion exchange chromatography on DEAE-Sephacel (Pharmacia Fine Chemicals AB, Uppsala, Sweden), column size 1.6×12 cm eluted with 0.0175 mol/l phosphate buffer, pH 6.8 at +4°C (flow-rate 20 ml/h), 5 ml fractions were collected. After the first protein peak (IgG's) was completely eluted, the elution buffer was changed gradually to 0.3 mol/l phosphate buffer, pH 6.5 by a gradient mixer. The fractions were tested for their penicillinase inhibitor activity. The positions of BSA, IgG_1, IgG_2 and IgM were determined. The function of the elution gradient was tested by analysing the phosphate content of the fractions by the phosphomolybdate method of *Goldenberg & Fernandez* (1966).

Determination of the minimum inhibitory concentration values (MIC)

Six IgG fractions from inhibitor positive cows were used to test their effect on the MIC-value for 1 penicillinase-producing and 1 penicillin sensitive strain of S. aureus. In 1 row of 12 wells of Microtiter plate 50 µl of IgG fraction and 50 µl of penicillin dilution (double dilution series) were mixed and 50 µl of S. aureus culture was added to all the wells. In the control row the IgG-fraction was replaced by elution buffer (0.0175 mol/l phosphate buffer, pH 6.8). The Microtiter plates were incubated at 37°C for 20 h and any turbidity of the wells observed visually.

RESULTS

When bovine sera containing penicillinase inhibitor activity were fractioned by gel filtration chromatography (Fig. 1) and ion exchange chromatography (Fig. 2), the inhibition activity was mainly located in the IgG-fraction and occasionally there was slight activity in the IgM-fraction; this means that the inhibition should be considered due to an antibody.

The prevalence of serum penicillinase inhibitor in different bovine groups is shown in Table 1. The frequency of serum

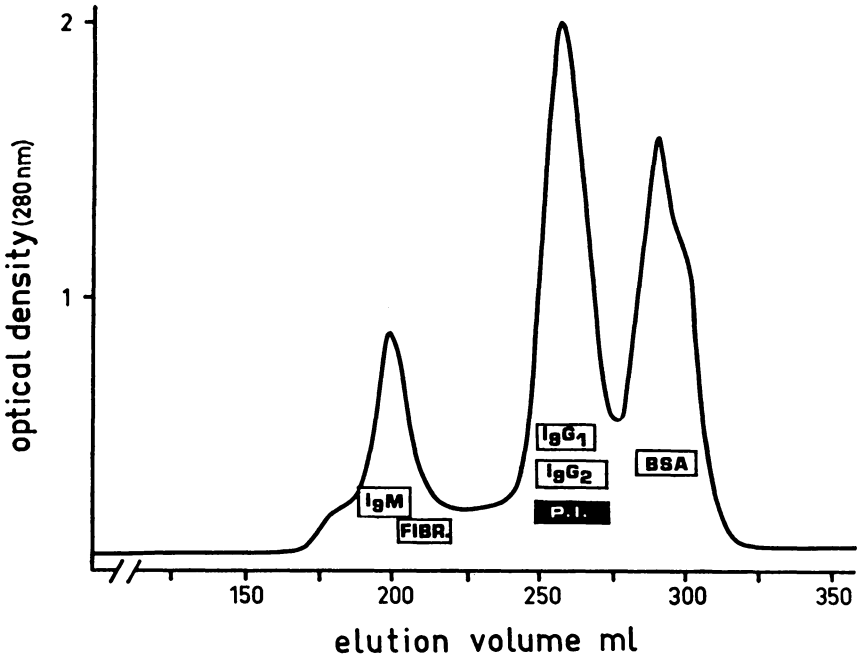

Fig. 1. Gel filtration chromatography of bovine serum on Sephacryl S-300. The location of penicillinase inhibitor is indicated — P.I. Note that P.I. is co-eluted with the IgG's.

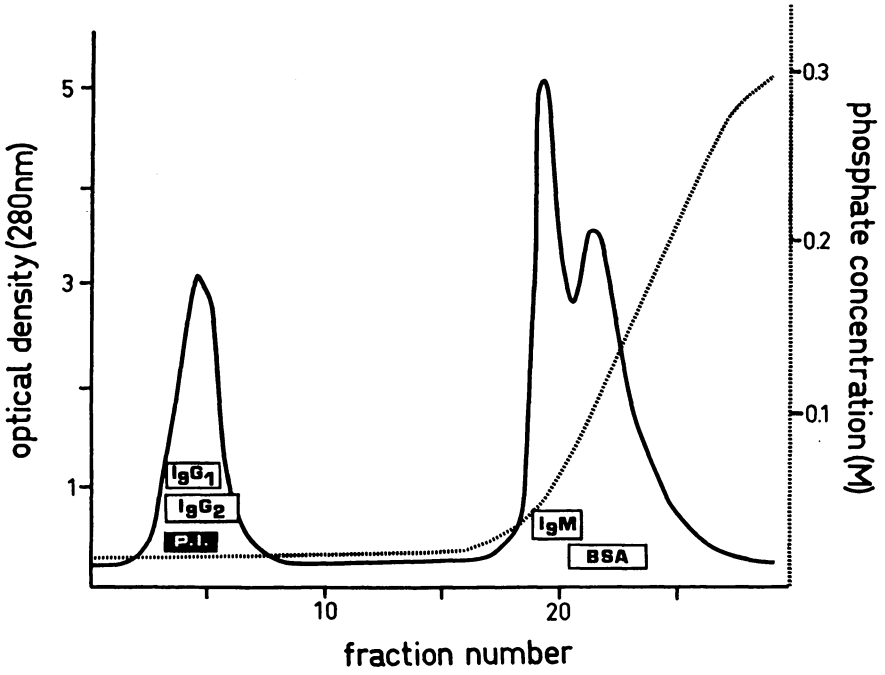

Fig. 2. Ion exchange chromatography of bovine serum on DEAE-Sephacel. Penicillinase inhibitor (P.I.) is eluted with the IgG-fraction.

Table 1. Presence of penicillinase inhibitor in different bovine groups.

	Number of sera			
	total	penicillinase inhibiting	non-inhibiting	Positive %
Mastitic cows	24	15	9	63
Healthy cows	10	0	10	0
Heifers	10	1	9	10
Calves	10	3	7	30

penicillinase inhibitor was highest in mastitis cows (63 %). The presence of serum penicillinase inhibitor as correlated with milk bacteriology is shown in Table 2.

When the MIC value for penicillin on a penicillin resistant S. aureus was tested with the presence or absence of penicillinase-inhibiting IgG-fraction from 6 cows, 5 decreased MIC for penicillin to one half (from 0.8 to 0.4 I.U./ml) and in 1 fraction no effect was seen. In the case of the penicillin sensitive strain of S. aureus no effect on MIC-value (0.003 I.U./ml) was seen in the same IgG-fractions.

Table 2. Serum penicillinase inhibitor activity as correlated with milk bacteriology.

		Number of sera		
	Milk bacteriology	total	penicillinase inhibiting	non-inhibiting
Clinical mastitis (n = 24)	Staphylococcus aureus	14	9	5
	Staphylococcus sp.	2	2	0
	Streptococcus sp.	1	1	0
	Escherichia coli	1	0	1
	negative bacteriology	6	3	3
Healthy control (n = 10)	negative bacteriology	10	0	10

DISCUSSION

Resistant bacteria present a problem in the clinical use of penicillin. The penicillin resistance of staphylococci is mediated by the production of β-lactamase (penicillinase) by the resistant

strains. The β-lactamases are bacterial enzymes which catalyze the rapid hydrolysis of the β-lactam ring of penicillins and cephalosporins. The manufacture of penicillinase by staphylococci is mediated by plasmids that can be transferred from one staphylococcus to another by bacteriophages (transduction) (*Richmond* 1965 b).

Lie & Solbu (1980) described penicillinase inhibitor activity in bovine sera. The characterization of these inhibitors was undertaken in the present study.

Separation of bovine sera, containing penicillinase inhibitor activity, by gel filtraton chromatography as connected by the analysis of penicillinase inhibitor capacity of each fraction indicated that the inhibitors were co-chromatographed in the fractions containing IgG_1 and IgG_2 (Fig. 1). Ion exchange chromatography confirmed that the inhibition is principally included in the IgG's (Fig. 2).

Theoretically it can be assumed that a presence of penicillinase inhibitor could act in synergy with β-lactam antibiotics to lower the minimum inhibitory concentration (MIC) towards resistant bacteria. Antisera to β-lactamase preparation have been prepared by immunising experimental animals with β-lactamase preparations (*Cole* 1979). It has been shown that such antisera could protect benzylpenicillin from inactivation by β-lactamases (*Perlstein & Liebman* 1945). *Housewright & Henry* (1947) showed that anti-β-lactamase halved the MIC for penicillin against penicillinase producing strain of S. aureus. However, *Richmond* (1975) illustrated the effects of specific antisera on β-lactamase activity, including inhibitory and stimulatory effects. Such results including possible activation would indicate that such antisera cannot be used therapeutically.

The method of determining penicillinase inhibitor activity used in the present study, picked up sera showing inhibitor activity but does not indicate possible activation. However, the presence of penicillinase inhibitor activity in different bovine groups indicate that the presence of the penicillinase inhibiting activity is a common phenomenon in cows. The high frequency of this inhibition in mastitic cows indicates that the production of this inhibitor could be due to immunostimulation by mastitic pathogens. The nature of these inhibitor molecules (IgG's) support this idea. Penicillinase inhibitor activity was seen in some mastitic cows without staphylococcal mastitis, indicating that

during the course of mastitis, staphylococci could have been present even if not isolated by this single bacteriological examination. Penicillinase inhibitor was present in 3 out of 10 calves. This can be explained by the origin of passive immunoglobulins from the cow due to colostral-intestinal "transfusion". To confirm this, samples should have been taken from a number of calves and their mothers parallelly. Another explanation could be that the calves had been infected with staphylococci subsequently.

To test the possible effect of the penicillinase inhibition in clinical applications, a MIC determination was carried out by 1 penicillinase resistant and 1 sensitive strain in the presence and absence of IgG-fractions (containing inhibitory activity) from 6 cows. In the case of β-lactamase producing staphylococci, the MIC was halved by 5 of the 6 inhibitor-fractions; on the other hand, no effect was seen on the sensitive strain. The present results clearly indicate an immunologically mediated production of penicillinase inhibitors. The slight effect on MIC indicates that the inhibition may have some influence in the clinical situation. The utilization of this inhibitor in diagnosis of staphylococcal infections such as mastitis requires further evaluation.

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SAMMANFATTNING

Karakterisering av penicillinas inhibitor i bovint serum.

Lie & Solbu (1980) beskrev penicillinas inhibitor aktivitet i bovint serum. Sera innehållande penicillinas inhibitor aktivitet fraktionerades med gelfiltration och jonbyteskromatografi. Penicillinas inhibitorn lokaliserades till IgG-fraktionen och kan därför anses vara en antikropp.

Vid en analys av 54 djur uppvisade mastitkor en högre insidens av antipenicillinas aktivitet än friska djur.

Penicillinas inhibitorn hade en lindrig effekt på MIC-värden för penicillin mot penicillinasproducerande stafylokokker, vilket betyder att inhibitorn kunde ha en klinisk betydelse genom att öka penicillinkänsligheten hos stafylokokkerna.

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Reprints may be requested from: Tuula Honkanen-Buzalski, the Department of Pharmacology and Toxicology, College of Veterinary Medicine, P. O. Box 6/Hämeentie 57, SF-00551 Helsinki 55, Finland.