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THE EFFECTS OF PEPTIDE-PRECIPITATING LIGNOSULPHONIC ACIDS ON THE PROTEOLYTIC ACTIVITY OF PEPSIN IN VITRO AND ON THE RESPONSE OF PIGS TO AN ULCER INDUCING DIET

By

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NÆSS, BJØRN, OLA WESTBYE, KJELL IVAR HILDRUM and INGER NAFSTAD: *The effects of peptide-precipitating lignosulphonic acids on the proteolytic activity of pepsin in vitro and on the response of pigs to an ulcer inducing diet.* Acta vet. scand. 1973, *14*, 44—56. — The inhibitory effect of peptide-precipitating lignosulphonic acids on the proteolytic activity of pepsin in vitro has been studied. When an unfractionated lignosulphonic acid preparation was used, total inhibition of the activity of 5 μg crystalline pepsin was obtained by addition of about 15 mg of lignosulphonic acids.

The greatest inhibitory effect was seen with the high-molecular weight lignosulphonic acids, but an effect was also seen with middle- and low-molecular weight lignosulphonic acids.

A reduction in the inhibitory effect was seen when the experiments were performed in the presence of arginine. The inhibition mechanism is discussed in relation to the peptide-precipitating ability of the lignosulphonic acids.

No obvious protection against the appearance of stomach ulcers in four pigs receiving an ulcerogenic diet was seen.

lignosulphonic acids; proteinase inhibition; pepsin; pig; ulcer inducing diet.

It has been demonstrated previously that swine pepsin in certain concentrations is inactive in agar gel containing neopeptone when lignosulphonic acids are present (*Næss* 1971b). This result is in accordance with the findings of *Vocac & Alphin* (1968) who used hemoglobin as substrate and in addition found that orally administered lignosulphonic acids protect pyloric-

ligated rats against the development of experimental gastric ulcers. This latter result is in agreement with the findings of *Fletcher et al.* (1957) who found a similar protective effect when the lignosulphonic acids were administered intraperitoneally to the pyloric-ligated rats.

The aim of the present work was to study the effects of certain peptide-precipitating lignosulphonic acids on the proteolytic activity of pepsin in vitro and on the occurrence of gastric ulcers in pigs receiving the ulcerogenic diet of *Nafstad* (1967). This diet has been shown to induce gastric ulcers in pigs to a great extent (80—90 %). It was assumed in the present work that pepsin is of importance for the occurrence of ulcers caused by this particular diet.

MATERIAL AND METHODS

Lignosulphonic acids. The sodium salt of lignosulphonic acids, prepared by the procedure of *Jantzen* (1967), was kindly supplied by Alwatech A/S, Oslo, Norway. The sulphur content of the lignosulphonic acids was 6 %.

Gel chromatography of the lignosulphonic acids. Fractionation was carried out by passing 1.5 ml of a 10 % solution of sodium lignosulphonate in 0.25 M-$CaCl_2$ through a column 25 mm in diameter and 100 cm long. Sephadex* G-50, fine, was used in ascending chromatography. The flow rate was adjusted to 32 ml per hr. and a 0.25 M-$CaCl_2$ solution used as eluant. Packing, stabilization and sample application were performed according to standard procedures (*Pharmacia* 1966). The effluent was collected in 60 10-ml fractions. For the analysis of any inhibitory effect on the proteolytic activity of pepsin, the fractions were combined 10 by 10 to give four 100-ml samples (fractions 17—56). In order to obtain sufficient material the same run was performed four times and the fractions combined as indicated above. The four combined samples from the four runs were dialyzed against running tap water for 18 hrs., evaporated in a Rotavapour** at 70°C, the dry weights estimated, and the fractions dissolved in 3 ml of distilled water. The four combined samples (fractions 17—26,

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* Pharmacia, Uppsala, Sweden.

** Buchi Rotavapour® Flawil, Switzerland.

27—36, 37—46 and 47—56) are hereafter called samples 1, 2, 3 and 4, respectively. Sample 1 contains mainly high-molecular weight lignosulphonic acids, samples 2 and 3 lignosulphonic acids of middle-molecular weight and sample 4 low-molecular weight lignosulphonic acids (*Hildrum & Næss* 1972).

Determination of lignosulphonic acids. The lignosulphonic acids in the fractions were determined using a method described by *Pearl & Benson* (1940), based on the principle that ligninous substances participate in a colour intensifying reaction on the addition of sodium nitrite. The intensity of the developed brown colour was measured at 430 nm using a Beckman* DB Spectrophotometer. The untreated fractions were used as blanks.

Estimation of the peptide-precipitating ability of lignosulphonic acids. To 2.5 ml of each fraction, 1 ml of 1 % neopeptone** in 0.1 M-HCl-KCl buffer, pH 2.1, or 1 ml of 1 % bovine hemoglobin (Sigma*** Type II, Lot H 121 B-241) in 0.1 M-HCl-KCl buffer, pH 2.1, was added, and the resulting turbidity measured at 700 nm. The untreated fractions were used as controls. To study the amount of protein-lignosulphonic acid precipitate formed in the Anson procedure (see below), pepsin was not added and the precipitate was centrifuged at 2500 $\times$ g for 10 min. The precipitate was washed once in 0.06 M-HCl and, after re-centrifugation and removal of the supernatant, dried overnight at 50°C and weighed.

To study the precipitation of pepsin by lignosulphonic acids at various pH values 1 ml 0.06 M-HCl containing 10 mg of lignosulphonic acids was added to 5 ml of a 0.2 % pepsin solution after adjusting the pH of the pepsin solution with concentrated HCl. The turbidity was measured at 700 nm.

Analysis of pepsin proteolysis inhibition. The effect of lignosulphonic acids on pepsin proteolysis in vitro was determined by the Anson method (*Anson* 1938), mainly as modified by *Vocac & Alphin* (1968), using bovine hemoglobin (Sigma) in 0.06 M-HCl as substrate. This method was found to be sufficiently accurate and sensitive for the present study.

The concentration of hemoglobin used in this work was, unless otherwise stated, 0.225 %. One ml of pepsin (ex hog stomach

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* Beckman Instruments Inc., Fullerton, California, USA.
** Difco Laboratories Inc., Detroit, Michigan, USA.
*** Sigma Chemical Compagny, St. Louis, Missouri, USA.

mucosa, 3 × cryst., Batch No. 44616*), 5 µg per ml, in 0.06 M-HCl or in various concentrations of L-arginine**, pH 2.1, and 1 ml of lignosulphonic acids at various concentrations in 0.06 M-HCl were added to 5 ml of substrate and incubated at 35 °C for 15 min. Five ml 0.3 M trichloroacetic acid was added, the solution centrifuged and 10 ml 0.5 M-NaOH and 3 ml Folin-Ciocalteus Phenol-reagent (Merck***) were added to 5 ml of the supernatants. The absorbances were measured at 480 nm. For the determination of peptic activity in stomach juice, 5 ml of substrate was added to 1 ml centrifuged stomach juice, and the procedure described above followed.

LD50 examinations. LD50 examinations of the lignosulphonic acids were performed on male rats, weighing 160—180 g by administering the lignosulphonic acids orally (*Pharmacopoea Nordica* 1970).

Pigs. Weaned Norwegian Landrace pigs of both sexes, randomly selected from several sources, and initially weighing from 20 to 30 kg, were used.

Diet composition. During the last 11 days before the experiment started, the pigs were gradually, from conventional feed, adapted to the final diet which consisted of: Casein, 16 % (Norsk Kasein A/L); cod liver oil†, 10 %; sucrose††, 38 %; potato starch†††, 32 %. Vitamins were supplied as follows (mg per 100 kg feed): Ascorbic acid, 500; nicotinic acid, 1600; calcium pantothenate, 1100; inositol, 400; choline, 3500; riboflavin 200; biotin, 20; folic acid, 70; pyridoxine, 130; thiamine hydrochloride, 200; vitamin B_{12}, 1. Mineral premix, 4000 g per 100 kg feed, was added, the premix containing in per cent: Dicalcium phosphate, 68; sodium chloride, 15; potassium chloride, 10; magnesium carbonate, 5; ferrous sulphate, 1.5; manganese sulphate, 0.25; copper sulphate, 0.1; cobalt chloride, 0.05; potassium iodide, 0.05; zinc oxide, 0.05 %. In addition to this feed, the four pigs were given 500, 1000, 2000 and 5000 g of lignosulphonic acids per 100 kg feed respectively (Table 1).

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* Koch-Light Laboratories, Colnbrook, Bucks., England.
** Nutritional Biochemicals Corp., Cleveland, Ohio, USA.
*** Merck, Darmstadt, Germany.
† Peter Møller, Oslo, Norway.
†† Tate & Lyle, London, England.
††† AL Opland og Toten Potetmelfabrikker, Norway.

Table 1. Start and final weights of the pigs, daily weight gain, amount of lignosulphonic acids given, pepsin activity of the stomach contents, and necropsy findings.

Pig no.	Lignosulphonic acids added (g per 100 g feed)	Total amount of lignosulphonic acids given during the experimental period (g)	Average amount of lignosulphonic acids given per day in the experimental period (g)	Body weight when the experiment started (kg)	Body weight when necropsied (kg)
1	0.5	468	8.1	21	56
2	1.0	415	13.8	20.5	33*
3	2.0	2440	42.1	29	79
4	5.0	5275	90.9	26.5	66

Table 1 (continued).

Pig no.	Average daily weight gain (kg)	Pepsin activity in 1 ml centrifuged stomach content after necropsy (equivalent to crystalline pepsin in μg)	Ulcers and scars in pars oesophagea of the stomach
1	0.60	10	one ulcer (10 by 1 cm)
2	0.42	8	one ulcer (6 by 6 cm)
3	0.86	12	three erosions (1 by 0.5 cm) (1.5 by 0.5 cm) (1 by 0.5 cm)
4	0.68	12	one erosion (2.5 by 1 cm) one scar

* Pig no. 2 died four weeks after the start of the experiment as a result of a gastric ulcer hemorrhage.

Five-hundred g of lignosulphonic acids per 100 kg feed, when fed to pigs, corresponds to the amount of lignosulphonic acids (50 mg/175 g body weight) giving 80—90 % protection against the development of gastric ulcers in pylorus-ligated rats (*Vocac & Alphin*) calculated on the basis of body weight.

Housing and feeding. The experiments were conducted within a closed building, the pigs were confined in a pen, with arrangements for individual feeding. The pigs were hand-fed twice a day and given adequate water supply. The animals were weighed once a week and killed 58 days after the start of the experiment, after which necropsies were performed.

RESULTS

Fig. 1 presents the results of the gel chromatography on Sephadex G-50 of the lignosulphonic acids used in this work using the procedure of *Pearl & Benson* (1940) for the determination of lignosulphonic acids. The turbidity, measured after adding bovine hemoglobin or neopeptone to the fractions, at pH 2.1, is also shown. It can be seen that the amount of precipitation varies with the different fractions. The neopeptone is precipitated mainly by the ligonsulphonic acids contained in the first peak (high molecular weight), while the hemoglobin is precipitated mainly by the lignosulphonic acids contained in the second peak (middle molecular weight).

In Fig. 2, the inhibition of pepsin proteolysis as a function of the amount of unfractionated lignosulphonic acids added is shown. Total inhibition of the activity of 5 μg crystalline pepsin is obtained, when about 15 mg of lignosulphonic acids are added. The inhibition seems to be reduced when the amount of lignosulphonic acids added exceeds 15 mg. A substantial reduction

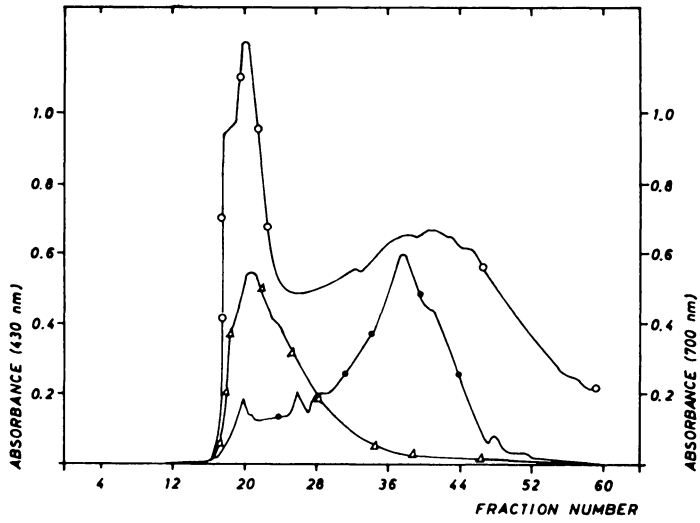

Figure 1. o—o Gel chromatography of the lignosulphonic acids on Sephadex G-50 assayed spectrophotometrically at 430 nm according to *Pearl & Benson* (1940).

△--△ Absorbance (at 700 nm) caused by the neopeptone-lignosulphonic acid precipitate in each fraction.

•—• Absorbance (at 700 nm) caused by the hemoglobin-lignosulphonic acid precipitate in each fraction.

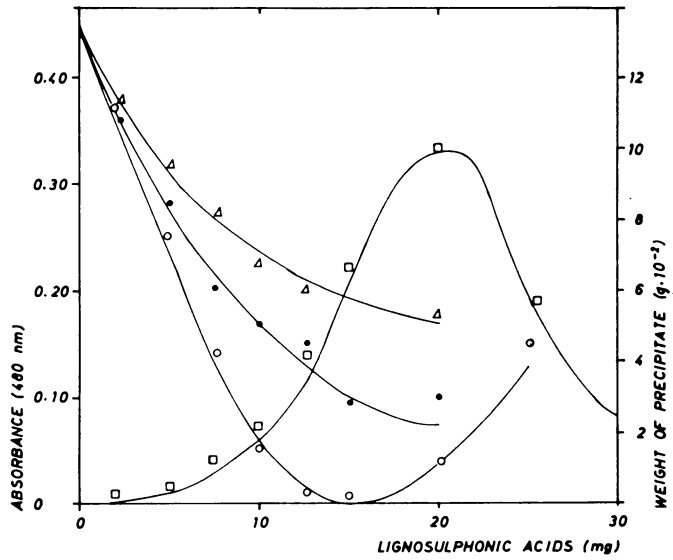

Figure 2. □—□ Weight of precipitate formed in the absence of pepsin using the *Anson* (1938) procedure after adding lignosulphonic acids to the solution.
○—○ Inhibition of the proteolytic activity of pepsin on adding unfractionated lignosulphonic acids.
Inhibition of the proteolytic activity of pepsin on adding unfractionated lignosulphonic acids in the presence of 0.25 M arginine •—•, and in the presence of 0.5 M arginine △—△.

in the inhibiting ability of the lignosulphonic acids is obtained when arginine is present in the mixture. The precipitation of hemoglobin in the absence of pepsin is also plotted in Fig. 2. It can be seen that the precipitation is at a maximum when about 20 mg lignosulphonic acids are added.

The inhibitory effect of lignosulphonic acids on pepsin proteolysis seems to be dependent on the molecular sizes of the lignosulphonic acids, to some extent, as can be seen from Fig. 3. The greatest inhibiting ability is observed for the lignosulphonic acids in the first peak (sample 1), but an inhibitory effect can also be seen for the other samples.

In Fig. 4 the inhibition of the proteolytic activity of pepsin with varying amounts of substrate is shown. It can be seen that the inhibition is strictly dependent on the concentration of hemoglobin. Pepsin is precipitated by lignosulphonic acids only at

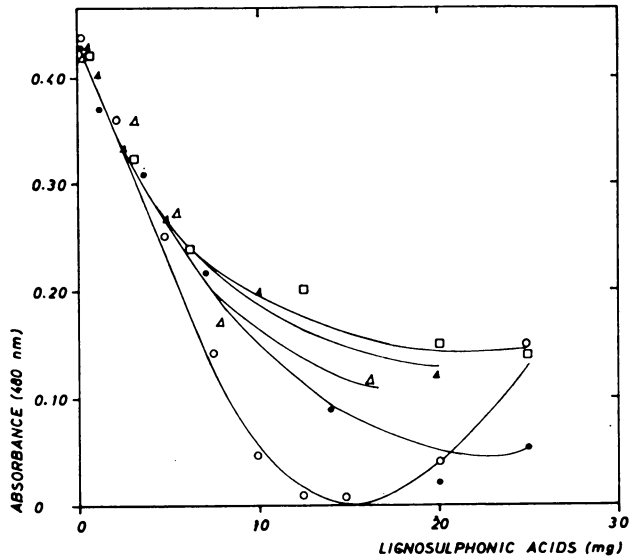

Figure 3. Inhibition of the proteolytic activity of pepsin on adding unfractionated lignosulphonic acids ○—○, and on adding fractions 17—26 from Fig. 1 ●—●, fractions 27—36 ▲—▲, fractions 37—46 △—△, and fractions 47—56 □—□.

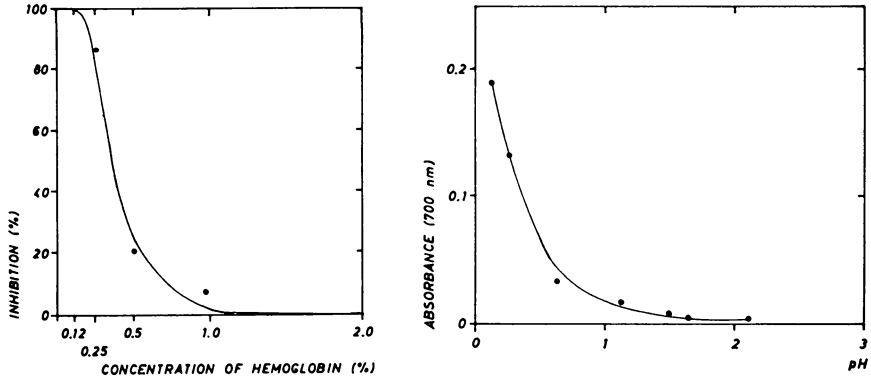

Figure 4. Inhibition of the proteolytic activity of 5 μg pepsin on adding 10 mg of lignosulphonic acids and varying the concentration of hemoglobin.

Figure 5. Absorbance at 700 nm caused by pepsin-lignosulphonic acid precipitates assayed at various pH-values.

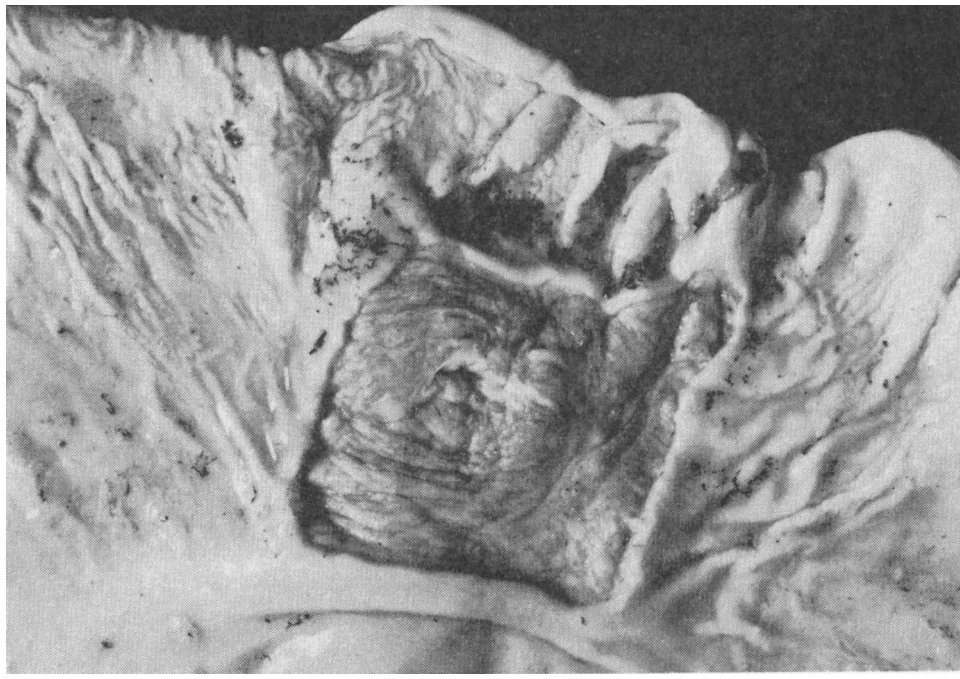

Figure 6. Gastric ulcer in the oesophageal region of the stomach of pig no. 2.

very low pH values as shown in Fig. 5. At pH 2.1 no precipitation of pepsin was observed.

LD50 in the male rats for the lignosulphonic acids used was found to be > 15 g per kg body weight.

The results of the feeding experiments with pigs are presented in Table 1. Pig no. 2 died four weeks after the start of the experiment as a result of hemorrhage from a gastric ulcer about 6 by 6 cm in the oesophageal region (Fig. 6). The pathological changes in the stomach of this pig and of the three other pigs in the experiment corresponded to those described by *Nafstad* (1967). The ulcers appeared as deep chronic lesions with thickened margins and formation of connective tissue in the base of the ulcer. The erosions were superficial lesions surrounded by irregularly thickened epithelium, which on histological examination appeared to be hyperkeratotic and parakeratotic.

DISCUSSION

The inhibitory effect of lignosulphonic acids on the proteolytic activity of pepsin in vitro is in agreement with the findings of *Vocac & Alphin* (1968). A modified *Jantzen* procedure (1967) for the preparation of lignosulphonic acids was used by those authors resulting in an average molecular weight for the lignosulphonic acids of approx. 5000 (low molecular weight). The sulphur content was approx. the same in the study of *Vocac & Alphin* as in the present work. An inhibitory effect of the low-molecular weight lignosulphonic acids was also found in the present work, but the greatest effect was, however, observed with the high-molecular weight lignosulphonic acids. It is possible that part of the inhibitory effect on the proteolytic activity of pepsin is due to the peptide-precipitating ability of the lignosulphonic acids. The blocking of the sulphonic acid groups with arginine (*Næss* 1971c) which resulted in a reduced inhibitory effect seems to support this theory. As the pepsin is not precipitated by the lignosulphonic acids at the pH value used (Fig. 5), the precipitating effect is with the substrate (the proteins are precipitated by lignosulphonic acids when the pH is below the isoelectric point of the proteins (*Næss* 1971a); the pI of pepsin is less than 1.0 (*White et al.* 1968)). On the other hand, the inhibitory effect was seen for all the samples of lignosulphonic acids tested, while the hemoglobin was precipitated mainly by the third sample (Figs. 1 and 3). Thus, factors other than the peptide-precipitating ability also seem to be of importance for the inhibitory effect. A possible explanation could be that non-precipitating lignosulphonic acids form soluble complexes with the proteins and, in this way, inhibit the proteolytic activity of pepsin. It is uncertain, however, whether this possible effect is upon substrate only or upon substrate and enzyme. *Vocac & Alphin* assume that the sulphur content and molecular weight of the lignosulphonic acids are of particular importance for the inhibition of pepsin proteolysis, and the authors conclude that the lignosulphonic acids act as a competitive inhibitor of pepsin proteolysis, and is more specific in action than most previously described compounds. As the inhibition is strictly dependent on the concentration of substrate (Fig. 4) and also seems to be related to the precipitation of the substrate, and as the enzyme is less affected by precipitation with the lignosulphonic acids

(Fig. 5), it seems incorrect to use the term competitive inhibitor about the lignosulphonic acids used in this study. It is also uncertain whether the term inhibitor should be used. In this connection it may also be mentioned that *Levey & Sheinfeld* (1954) have studied the inhibition of the proteolytic action of pepsin by sulphate-containing polysaccharides, including heparin. Heparin and the lignosulphonic acids react similarly with many proteins, forming complexes under certain conditions. The authors suggest that the inhibition of pepsin proteolysis is due to the formation of a complex between the enzyme and the polysaccharide which has less activity, or which reduces the amount of free pepsin available at any time.

Contrary to the results of the in vivo experiments of *Vocac & Alphin* and of *Fletcher et al.* (1957), no obvious protection against stomach ulcers was found in the present work, as all the pigs used showed severe changes in the oesophageal region of the stomach. As the inhibition seems, to a great extent, to be dependent on the concentration of lignosulphonic acids, this may have been too low or too high for the inhibition of pepsin proteolysis in this experiment. Peptic activity was demonstrated in the stomach juice after necropsy on the four pigs (Table 1). The dramatic result with pig no. 2 may also be seen in relation to the significant anticoagulant effect of the lignosulphonic acids reported by *Loomis & Beyer* (1953) and by *Fletcher et al.* Gastric ulcers, in the presence of an anticoagulant, probably lead to an increased mortality rate. The possibility of using lignosulphonic acids in the treatment of peptic ulcers should be considered in relation to the in vivo antipeptic effect on the species of interest. The hazards of allowing a substance with anticoagulant activity to come into contact with a potentially hemorrhagic lesion should also be considered in this connection. It also seems important that antipeptic and anticoagulant effects should be considered in connection with the use of sulphite spent liquor as adhesives in the pelletizing of animal feeds. Although this utilization is only a few years old, it already ranks second to that of oil-well-drilling mud additives in the amount of lignosulphonates employed in USA (*Pearl* 1967). The antipeptic and anticoagulant effect should also be considered in connection with the use, as animal feed, of dried protein-lignosulphonic acid precipitates derived from protein-containing waste waters as proposed by *Jantzen*. In addition, these effects should be seen in connection

with the use of sulphite spent liquor powder as a food additive (*Pietz* 1967). The combination of antipeptic and anticoagulant effects is also known for substances other than lignosulphonic acids, for example carrageenin which is a sulphated galactan (*Hawkins & Leonard* 1962).

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SAMMENDRAG

Virkningen av peptidpresipiterende ligninsulfonsyrer på proteolytisk aktivitet av pepsin in vitro og på griser fôret med en magesårfremkallende diett.

En har studert virkningen av peptidpresipiterende ligninsulfonsyrer på proteolytisk aktivitet av pepsin in vitro. Når ufraksjonerte ligninsulfonsyrer ble brukt, fikk en fullstendig inhibering av 5 µg krystallinsk pepsin ved tilsetting av 15 mg ligninsulfonsyrer.

Den største inhiberende virkning fant en med de høgmolekylære ligninsulfonsyrer, men det ble også funnet inhiberende virkning av middel- og lavmolekylære ligninsulfonsyrer.

Det ble funnet en reduksjon i den inhiberende virkningen når arginin ble tilsatt. En har diskutert inhiberingsmekanismen i forhold til peptidpresipiterende egenskaper for ligninsulfonsyrer.

Det ble ikke funnet noen tydelig virkning av ligninsulfonsyrer på forekomst av magesår hos 4 griser som ble fôret med en ulcerogen diett.

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