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THE PRECIPITATION OF PEPTIDES AND PROTEINS BY LIGNOSULPHONIC ACIDS

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Lignin and lignosulphonic acids are among the main components of sulphite spent liquor, produced in the sulphite process for the manufacture of chemical wood pulp.

The chemical structures of the lignins are not clearly defined, but proposed formulations for lignins have been reviewed by *Pearl* (1967). Salts of lignosulphonic acids can be precipitated from cold aqueous solution in two main ways, according to *Borišek & Stanic* (1958): 1) by reaction with certain inorganic or organic substances, 2) by separation of the lignosulphonic acid molecules from the hydrate shell, and precipitation with respect to the colloid nature of the liquor.

The lignosulphonic acids react with certain proteins to form complexes, and this reaction has been utilized by *Jantzen* (1969), for the separation and isolation of certain lignosulphonic acids in sulphite spent liquors. The reaction is also used in tanning hides to leather (*Reich* 1967) and in removing contaminating proteins from the effluents of canneries and fish-processing plants (*Pearl* 1957) and from slaughter-houses (*Tønseth & Berridge* 1968, *Jørgensen* 1971). *Sieber* (1951) described the precipitation of gelatin, in NaCl-solution by lignosulphonic acids, as a characteristic qualitative reaction of sulphite spent liquor.

The aim of the present work was to study the precipitation of various peptides and proteins with sulphite spent liquor and with isolated lignosulphonic acids.

MATERIALS AND METHODS

Sulphite spent liquor. A fermented liquor from an alcohol distillery was used. The analytical data of the liquor were as follows: Dry basis 8.5 %; sugars 1.5 % and pH 5.1. This liquor is referred to as crude sulphite spent liquor. Dialysed liquor was also used with dialysis being carried out against running tap water for 15 hrs. The liquors were stored at -20°C.

Lignosulphonic acids. Sulphite spent liquor was treated with CaO (1.5 g CaO per 100 ml of sulphite spent liquor) at 40°C. The precipitate was centrifuged and washed twice with distilled water, after which the upper homogenous layer of the centrifuged precipitate was collected, dissolved in 0.01 M-HCl, and 0.1 M-NaOH added to pH 6.8. The solution was dialysed against running tap water for 15 hrs. in order to remove low molecular weight saccharinic acids and saccharates, and small free cations and anions according to the precipitation theory of *Hall* (1956). Low molecular weight lignosulphonic acids are also supposed to be lost during dialysis. Only traces of oxalic acid-precipitable substances could be identified after dialysis. The purified material was freeze-dried and stored in a desiccator at room temperature, and solutions to be used prepared when needed. These lignosulphonic acids are referred to as isolated lignosulphonic acids.

Agar gel. For experiments performed on agar plates 1.15 % Bacto-agar (Difco* 0140-01) was used as basis. Thimerosal was added (final concentration 0.01 %) as microbicidal agent. The addition of thimerosal was found to have no influence on the experiments carried out on the agar plates.

Preparation of peptide- and protein-agar plates. One of the following peptides or proteins was added to the melted agar at 70—80°C, to a final concentration of 1, 2 or 3 %: Sodium caseinate (Eastman Kodak**), neopeptone (Difco 0119-01), proteose-peptone (Difco 0120-01), gelatin (Gelita***), or a number of

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* Difco Laboratories Inc., Detroit, USA.

** Eastman Kodak, New York, USA.

*** Gelita, Göppingen, Germany.

dipeptides* (Sigma**). The hot mixture was then poured into horizontally placed Petri dishes to a thickness of 1.5 mm. Agar plates containing serum were prepared by adding horse serum to a concentration of 5 or 10 % (v/v). Agar plates, with various pH values, were prepared for each type of peptide and protein in the range pH 4—8, by adding 1 M-HCl or 1 M-NaOH. A 2 % neopeptone agar plate with dialysed peptides and pH 6.2 was also prepared, the dialysis carried out against running tap water for 15 hrs.

Circular wells of 7 mm diameter were cut in the solid agar with a cork borer, and 50 µl of the lignosulphonic acid-containing solutions to be tested were applied into the wells.

Isoelectric points. The isoelectric points of the peptides were determined by studying the migration of the peptides in agar gel electrophoresis. The agar gels were prepared as 1 % agar (Difco, Special Noble agar, 0142-01) in 0.05 M Tris-HCl buffers, the pH being varied from 6.6 to 8.0. The same buffers were also used as running buffers. The peptide solution (7 %) was applied in circular wells (2.5 mm diameter) in agar gel on microscope slides, and the electrophoresis carried out with LKB† equipment for gel electrophoresis. The running time was 60 min. at 300 v.

Developing system. For identifying the low molecular weight peptides after electrophoresis, a longitudinal ditch was cut in the agar gel and filled with ninhydrin in methoxyl ethanol solution (2 %). The slides were then incubated at 50°C for 2 hrs., the peptides being stained bluish-red by the ninhydrin. High molecular weight peptides were identified by drying parallel slides at room temperature overnight and staining with 1 % amido black 10 B (Merck††) in 7 % acetic acid followed by differentiating with 7 % acetic acid.

Spectrophotometric measurements. The turbidity caused by peptide- or protein-lignosulphonic acid complexes in agar gel was

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* DL-alanyl-DL-serine, DL-alanyl-DL-phenylalanine, L-alanyl-L-histidine, glycyl-L-methionine, glycyl-L-asparagine, glycyl-L-aspartic acid, L-valyl-L-leucine, glycyl-L-isoleucine, glycyl-L-proline, glycyl-L-alanine, N-glycyl-L-serine, DL-alanyl-DL-asparagine, DL-alanyl-DL-alanine, DL-leucylglycine, L-valylglycine, L-leucyl-L-serine.

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

† LKB Instruments, Ltd., Stockholm, Sweden.

†† Merck, Darmstadt, Germany.

measured at room temperature in a "Hilger"* spectrophotometer at 700 nm after mixing the reagents in melted agar at 70—80°C in standard test tubes and allowing the solution to solidify. Precipitation experiments in solution, in the absence of agar, were also carried out in standard test tubes and the turbidity measured as described.

Chemicals. The chemicals used were of analytical grade.

RESULTS

Isolated lignosulphonic acids, dialysed sulphite spent liquor and crude sulphite spent liquor, were separately applied into different wells in agar plates holding various pH values, and containing various concentrations of sodium caseinate, horse serum, neopeptone, proteosepeptone, gelatin or a number of dipeptides. After 15—30 min. at 25°C, distinct, circular yellow-grey or greyish-white precipitation zones appeared around the wells on neopeptone-, proteosepeptone-, gelatin-, caseinate- and serum-containing agar plates, primarily on most acid plates. Neopeptone resulted in the most distinct precipitation zones around the wells containing isolated lignosulphonic acids or dialysed sulphite spent liquor (Fig. 1). In order to obtain distinct precipitation zones, 2 % neopeptone in the agar was found suitable. Dialysis of the neopeptone before preparing the agar plate did not prevent the occurrence of precipitation zones.

When the diameter of the zones exceeded about 12 mm, in 2 % neopeptone agar plates, within 24 hrs. at 25°C, central zones of lysis appeared. Heating of the lignosulphonic acid solution to 100°C for 10 min. before application had no effect on the occurrence of zones of lysis.

When crude sulphite spent liquor was used, sodium caseinate agar plates gave the most distinct precipitation zones. The precipitating activity in this system was, however, reduced, after heating the sulphite spent liquor to 100°C for 10 min. before applying it into the agar wells. The remaining activity was, in addition, considerably reduced after dialysis. The Ca^{++} content in the crude sulphite spent liquor was found to be considerable.

The dipeptides tested did not result in precipitation zones in the pH region 4.0—7.5.

Fig. 2 shows precipitation zones caused by a serial 2-fold

* Hilger & Watts, Rank Precision Industries Ltd., London, England.

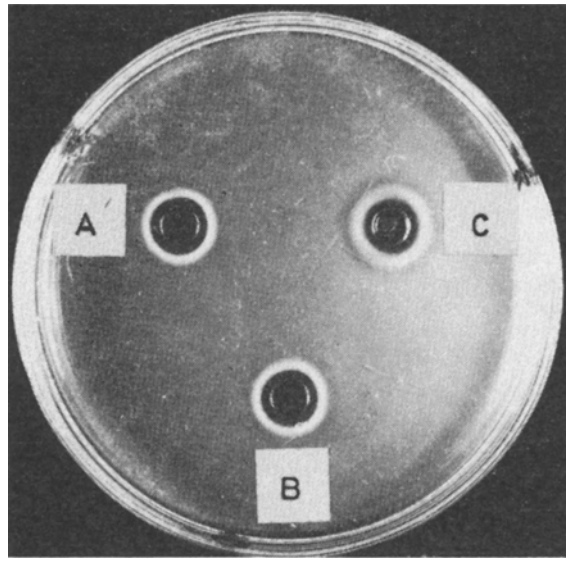

Figure 1. Precipitation zones caused by 50 µl 2 % isolated lignosulphonic acids (A), 50 µl dialysed sulphite spent liquor (B) and 50 µl crude sulphite spent liquor (C), in an agar plate containing 2 % neopeptone. pH in the agar and liquors 6.8. Incubation time 5 hrs. at 25°C.

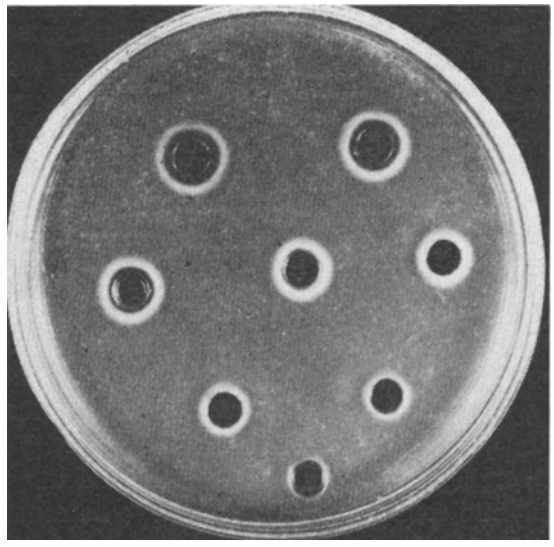

Figure 2. Precipitations zones caused by transferring aliquots of 50 µl of 2-fold serial dilutions of crude sulphite spent liquor to wells in an agar plate containing 2 % neopeptone, pH 6.8. The dilutions are from left to right in each row: 1/1, 1/2, 1/4, 1/8, 1/16, 1/32, 1/64 and 1/128. The incubation time was 4 hrs. at 25°C.

dilution of crude sulphite spent liquor in a neopeptone agar plate. As can be seen, the diameters of the zones are dependent on the concentrations of the lignosulphonic acids. The relation between the diameters of the precipitation zones and the incubation time, at various incubation temperatures, is shown in Fig. 3. It can be

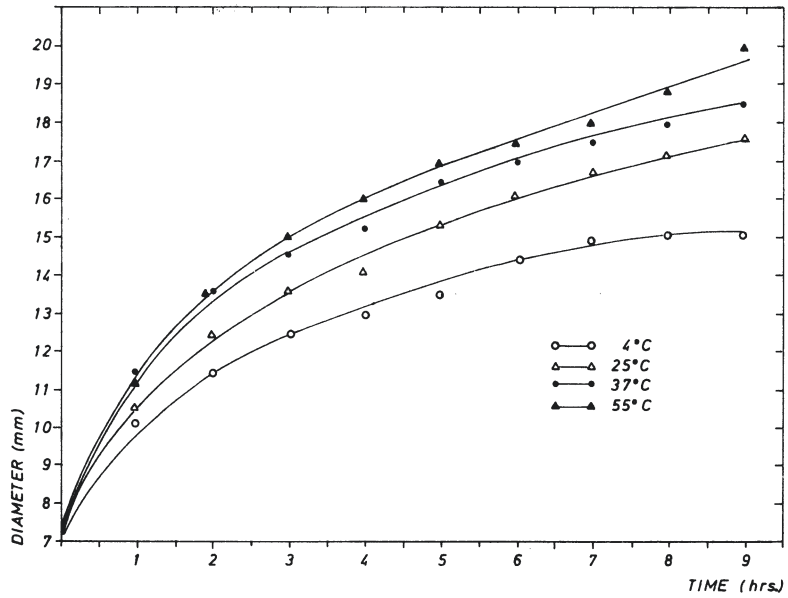

Figure 3. Diameters of precipitation zones in agar plates containing 2 % neopeptone, caused by 50 µl crude sulphite spent liquor, measured as a function of time. pH in agar and liquors 6.8.

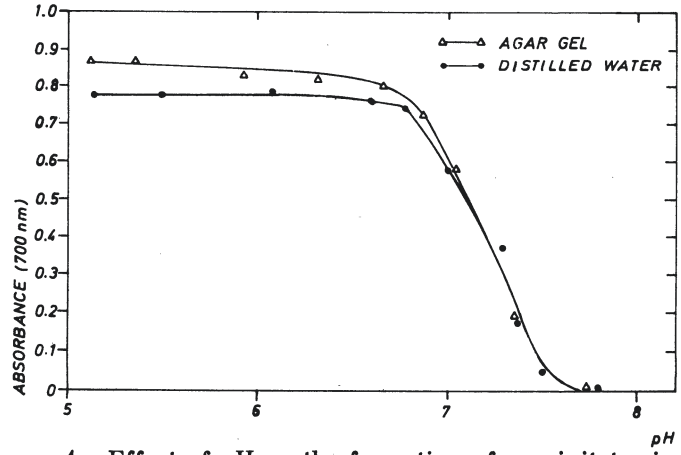

Figure 4. Effect of pH on the formation of precipitates in a mixture of lignosulphonic acids and peptides in distilled water, and in solid agar gel, measured spectrophotometrically. Reaction mixture: 2.0 ml distilled water or agar (1.4 %) containing 2 % neopeptone, and 0.2 ml dialysed sulphite spent liquor.

seen that when the plates were incubated at 4°C, 25°C, 37°C and 50°C respectively, the zones of precipitation increased with increasing temperature, provided that the other conditions were constant. The diameters increased gradually for incubation periods up to 24 hrs. at 25°C and the border of the zones remained quite distinct. Further incubation resulted in steadily increasing diameters, but the borders of the zones became gradually indistinct. The turbidity caused by peptide-lignosulphonic acid precipitates in solid agar gel and in distilled water was measured as absorbance at 700 nm and is plotted against pH (Fig. 4). The figure shows that the precipitation is strictly dependant on pH. An essentially similar pH dependency was found with, and without, agar in the reaction mixture. In order to obtain significant precipitation, the pH of the neopeptone agar plates was, therefore, in later experiments, adjusted to 6.8, the highest value giving maximum precipitation.

Precipitates of peptide- and protein-lignosulphonic acid complexes were found to be readily soluble in dilute NaOH, and insoluble in dilute HCl.

In Figs. 5 and 6 the turbidity caused by peptide-lignosulphonic acid complexes in distilled water and in solid agar gel is shown as a function of the lignosulphonic acid and peptide concentration respectively. It can be seen that the dependency curves have essentially similar shapes when the reaction mixtures contain, and do

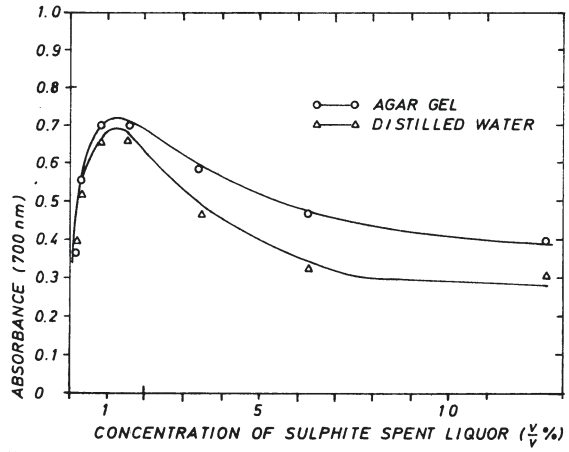

Figure 5. Absorbance caused by peptide-lignosulphonic acid precipitates in distilled water, and in solid agar gel (0.7 %) as a function of the crude sulphite spent liquor concentration in an 0.5 % neopeptone solution. pH in the mixture 6.8.

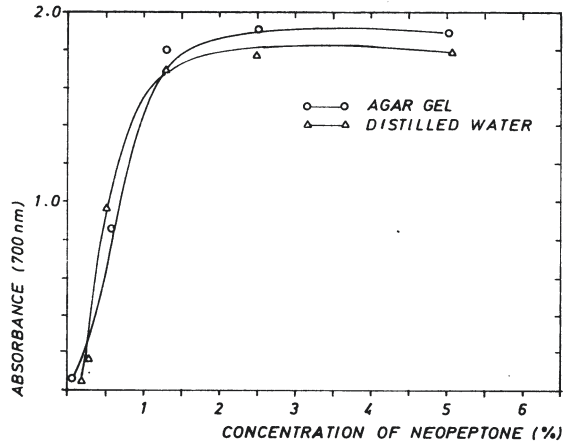

Figure 6. Absorbance caused by peptide-lignosulphonic acid precipitates in distilled water, and in solid agar gel (0.7 %), as a function of peptide concentration in an 0.3 % (v/v) solution of crude sulphite spent liquor. pH in the mixture 6.8.

not contain, agar. A relationship similar to that shown in Fig. 5, was found when isolated lignosulphonic acids were substituted for sulphite spent liquor in the reaction mixtures.

Isoelectric points for peptides in neopeptone were found to vary between pH 7.0 and 7.5.

Application of lignosulphonic acids into wells in agar plates not containing peptides or proteins, did not result in any zones of precipitation.

DISCUSSION

The agar did not seem to participate in the chemical reactions in these experiments, as similar results were obtained in solid agar-containing and liquid agar-free reaction mixtures. Thus peptide- and protein-containing agar plates were found suitable for the demonstration of, and studies on, the formation of peptide- and protein-lignosulphonic acid precipitates, and on reactions with other components of sulphite spent liquor. Except for dipeptides, precipitation zones were obtained with all the peptides and proteins tested. This result indicates that the peptides have to exceed a minimum molecular size in order to form precipitates with lignosulphonic acids.

Central lysis in the precipitation zones occurred only around the wells containing the most concentrated lignosulphonic acid

solutions (Fig. 2). Due to the thermostability of the lysis-forming components, the lysis zones do not seem to be caused by proteolytic enzymes possibly present in the liquors. It can be seen from Fig. 5 that excess of sulphite spent liquor in a peptide solution, inhibits the formation of peptide-lignosulphonic acid precipitates. This could be the explanation for the appearance of lysis zones in the peptide-lignosulphonic acid precipitation zones in the agar plates.

The temperature labile precipitating components of crude sulphite spent liquor which were found after application of the liquor into wells in caseinate agar plates, are assumed to be casein-precipitating enzymes (*Sandvik* 1962). As the Ca^{++} content in the crude sulphite spent liquor was found to be considerable, this may be among the dialysable components which influence the caseinate precipitation.

Borišek & Stanic (1958) regarded lignosulphonic acid as a molecular colloid with a negative charge due to the $—SO_3H$ groups and with a hydrate shell. Precipitation of this system may occur when the molecule is separated from the hydrate shell and the negative charge is neutralized. The conclusions of *Borišek & Stanic* were based on studies of the effect of lower alcohols and certain salts on lignosulphonic acids. It can be seen from Fig. 4 that in order to obtain precipitation in the presence of neopeptone and lignosulphonic acids, the pH in the solutions has to be below 7.5. The effect of pH on the precipitation considered in relation to isoelectric points of the peptides, as found in this work, seems to support the precipitation theory of *Borišek & Stanic*. A possible explanation of the precipitation of peptides and proteins by lignosulphonic acids may be: Parts of the peptides may form hydrogen bonds with the molecular colloid surface and thus have a separating effect on the hydrate shell, and positively charged amino-groups may neutralize negatively charged sulpho-groups. Another explanation may be: Parts of the peptides may form hydrogen bonds with the molecular colloid surface, and negatively charged carboxyl-groups may be linked to negatively charged sulpho-groups by bivalent metallic ions. The latter theory seems unlikely, however, as dialysis of the sulphite spent liquor and peptide solutions did not prevent the peptide-lignosulphonic acid precipitation and no significant oxalic acid-precipitate could be detected after dialysis. A theory based on work with collagen, as reviewed by *Reich* (1967), states

that the protein-lignosulphonic acid bond is primarily of electrostatic nature, but that hydrogen bonds may occur from hydroxyl groups in the lignosulphonic acid molecule to the peptides.

The effect of the incubation temperature and the initial concentration of lignosulphonic acids upon the formation of precipitation zones in agar, is in agreement with the general findings on diffusion of substances in agar gel (*Crowle* 1961).

Studies on the precipitating ability of lignosulphonic acids on peptides and proteins, are important in order to explain the chemical reactions which occur when sulphite spent liquors are released into fiord basins and rivers. Similar studies may also elucidate what happens in the liquor when sulphite spent liquors are used as substrates for microbial protein production (single cell protein production).

As the zone diameters seem to be dependent on the concentration of the lignosulphonic acids, the described agar diffusion method could possibly be developed as a direct micro quantitative procedure for the determination of peptide-precipitating lignosulphonic acids in aqueous solution.

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SUMMARY

The precipitation of peptide- and protein-lignosulphonic acid complexes can be demonstrated and studied in agar plates in the form of precipitation zones. The effects on the precipitates caused by variations in incubation temperature, incubation time, concentration of the reagents and in pH in the mixture of reagents, are described, and the nature of the peptide-lignosulphonic acid bond is discussed. The central lysis zones which appeared in some precipitation zones were found to be probably caused by excess of lignosulphonic acids. The possibility of developing the agar precipitation method described as a direct micro quantitative procedure for the determination of certain lignosulphonic acids in aqueous solution is suggested.

SAMMENDRAG

Presipitering av peptider og proteiner med ligninsulfonsyrer.

Det har vist seg mulig å demonstrere og studere presipitering av peptider og proteiner med ligninsulfonsyrer i form av presipiteringssoner i agarplater. En har beskrevet virkningen på presipitatet av variasjon i inkuberingstemperatur, inkuberingstid, konsentrasjon av reagenter og pH i blandingen av reagenter, og en har diskutert bindingen i peptid-ligninsulfonsyre-komplekset. Lysissonene som ble utviklet sentralt i noen presipiteringssoner, er trolig forårsaket av overskudd av ligninsulfonsyrer. Muligheten for å utvikle den beskrevne agar-presipiteringsmetode som en direkte mikro-kvantitativ metode for å bestemme visse ligninsulfonsyrer i vandig løsning er antydet.

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