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PEPTIDE-LIGNOSULPHONIC ACID PRECIPITATION ZONES IN AGAR GEL

A DIRECT MICRO QUANTITATIVE PROCEDURE FOR THE DETERMINATION OF PEPTIDE-PRECIPITATING LIGNOSULPHONIC ACIDS IN AQUEOUS SOLUTION

By
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It has been shown that agar plates containing certain peptides, under defined conditions are suitable for the demonstration of, and studies on, the formation of peptide precipitates with lignosulphonic acids (*Næss* 1971). By this method, characteristic, distinct yellow-grey precipitation zones appear around wells in agar plates into which lignosulphonic acids have been applied. The zone diameter was found to be dependent on the lignosulphonic acid concentration.

Many procedures for the determination of lignin and lignosulphonic acids in aqueous solution have been proposed, and are reviewed by several authors including *Ritter* (1967) and *Pearl* (1967). Most of the procedures reported are macro or semi-micro procedures. Many of them are based on the ability of certain substances to precipitate lignin or lignosulphonic acids. Among these precipitants are organic amines and a mixture of aniline and formaldehyde. Other procedures are based on the determination of the amount of methoxyl groups present in lignin and lignosulphonic acid molecules. Methods based on colour reactions, ultraviolet and infrared spectrophotometry have also been developed. The methods described seem to be hampered by the lack of calculable precision (*Ritter*) and do not distinguish clearly between lignin and lignosulphonic acids.

The aim of the present work was to study the relationship between zone diameter and lignosulphonic acid concentration when applying the peptide-lignosulphonic acid precipitation zone phenomenon in agar plates.

MATERIALS AND METHODS

Sulphite spent liquor. A fermented liquor from an alcohol distillery was used. Analysis of the liquor was as follows: Dry basis 8.5 %; sugars 1.5 %; pH 5.1. The sulphite spent liquor was kindly supplied by A/S Tofte Cellulosefabrik, Hurum, Norway.

The agar precipitation method. Agar, 1.15 % (Difco*, Bacto-agar, 0140-01) in water was used as basis. Thimerosal was added (final concentration 0.01 %) as a microbicidal agent. Neopeptone (Difco 0119-01) was added to the agar to a final concentration of 2 %. The melted peptide- and thimerosal-containing agar was poured into horizontally placed Petri dishes or rectangular glass plates measuring (12×18) cm. The frame of the plates was made of 0.6 cm thick and 2.0 cm broad perspex strips, which were fixed to the glass bottom plate by means of plastic tape. Tight-fitting glass plates served as covers. The volume was calculated to give an agar layer thickness of 1.5 mm. Fifty µl of the solutions to be tested were applied into circular wells of 7 mm diameter in the agar. The interval between the wells was 2—3 cm. The diameters of the precipitation zones were measured to the nearest 0.5 mm. The plates were usually used after 1 hr.'s storage at room temperature.

Definition. The dilution of the lignosulphonic acid solution corresponding to the point of intersection between the abcissa and the straight regression line (x-intercept) for zone diameter as a function of lignosulphonic acid dilution ($\log_{10}$) contains 1 diffusion unit per 50 µl liquor (Fig. 4).

Statistics. The standard deviation in the point of intersection (x) between the regression line and the abcissa was found from the formula

$$s_x = \sqrt{\left(\frac{\delta x}{\delta a} s_a\right)^2 + \left(\frac{\delta x}{\delta b} s_b\right)^2 + \ldots\ldots}$$

* Difco Laboratories Inc., Detroit, USA.

where $\frac{\delta x}{\delta a}, \frac{\delta x}{\delta b}$ designate partial derivatives, and a, b designate variables in the mathematical expression of x (*Bjørnes & Hovde* 1968). As the diameters of the agar wells were 7 mm, the point of intersection between abcissa and the straight regression line is defined by

$x = \frac{7 - a}{b}$ where a is y-intercept and b is the regression coefficient. The relative uncertainty in the x-intercept can thus be expressed by

$$\frac{s_x}{x} = \sqrt{\left(\frac{s_a}{7 - a}\right)^2 + \left(\frac{s_b}{b}\right)^2}$$

To test the linearity of semilogarithmic regression lines a standard regression analysis programme, based on conventional statistical methods, was run on a computer (IBM* 360/40).

Comparative lignosulphonic acid determinations. Parallel determinations of lignosulphonic acids were carried out according to a method desribed by *Ritter* (1967): 5 ml of sulphite spent liquor was added to 5 ml of a filtered solution of 3 % β-naphthylamine (Merck**) in 12 % HCl, and the mixture placed in a boiling water bath for 2 hrs. The precipitate was washed with distilled water, dried at 105°C, and weighed. The weight (a) was multiplied with the following empirical factors: a × 0.793 × 1.22 = lignosulphonic acids (g).

Chemicals. All the chemicals used were of analytical grade.

RESULTS

Neopeptone agar plates, with sulphite spent liquor applied in wells in the agar in serial 2-fold dilutions, were incubated for 9 hrs. at 4°C, 25°C, 37°C and 55°C, four parallel plates at each temperature. Fig. 1 shows a plate incubated for 7 hrs. at 25°C. The central lysis zones around some of the wells have been discussed previously (*Næss* 1971). The dependence of zone diameter on the dilution of the lignosulphonic acids at various incubation temperatures is shown in Fig. 2. The relationship between zone diameter and the lignosulphonic acid dilution ($\log_{10}$) at various

* IBM, World Trade Corporation, New York, USA.

** Merck, Darmstadt, Germany.

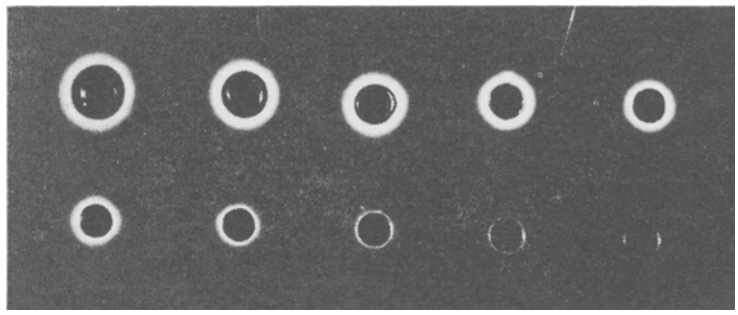

Figure 1. Precipitation zones in neopeptone agar corresponding to 2-fold serial dilutions of lignosulphonic acids. Incubation time 7 hrs. at 25°C.

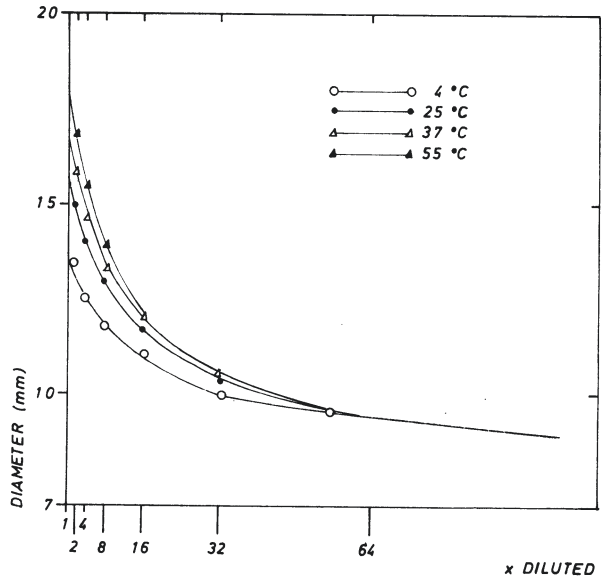

Figure 2. Influence of incubation temperature on the diameter of precipitation zones caused by lignosulphonic acids in serial 2-fold dilutions. Incubation time 6 hrs.

incubation times is presented in Fig. 3. To find the incubation temperature and incubation time which gave "the best straight" regression line, a regression analysis was carried out. The results are shown in Table 1.

Table 2 lists constants for estimated straight regression lines ($y = a + bx$). It can be seen from Table 2 that minimum relative uncertainty in the point of intersection between the straight

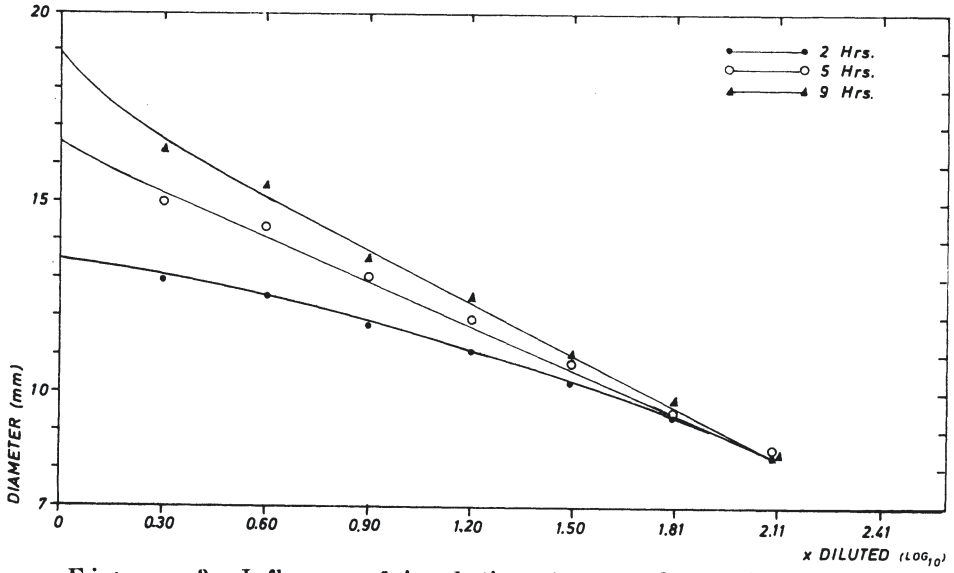

Figure 3. Influence of incubation time on the semilogarithmic regression curves for zone diameter as a function of lignosulphonic acid dilution. Incubation temperature 37°C.

regression line and the abcissa, under the conditions investigated, is found for an incubation of 6 and 7 hrs. at 37°C. The most suitable incubation conditions giving minimum relative uncertainty were, according to these results, chosen to be 7 hrs. at 37°C, when using the defined diffusion unit in the agar precipitation method.

Table 1. Curvature of the regression lines for zone diameters as a function of lignosulphonic acid dilution ($\log_{10}$).

Incubation time, hrs.	Incubation temperature			
	4°C	25°C	37°C	55°C
1	neg.*	neg.	neg.	n.s.**
2	neg.	neg.	neg.	neg.
3	neg.	neg.	neg.	n.s.
4	neg.	neg.	neg.	n.s.
5	neg.	n.s.	neg.	pos.***
6	neg.	n.s.	n.s.	pos.
7	neg.	pos.	n.s.	pos.
8	n.s.	pos.	pos.	pos.
9	n.s.	pos.	pos.	pos.

*: neg. = Significant negative curvature (at 5 % level).
**: n.s. = Not significant curvature (at 5 % level).
***: pos. = Significant positive curvature (at 5 % level).

Table 2. Analysis of estimated straight regression lines for zone diameter as a function of lignosulphonic acid dilution ($\log_{10}$).

Incubation temp., °C	Incubation time, hrs.	Coefficient of determination, r^2	y-intercept, a	Regression coefficient, b	Relative uncertainty s_x/x (%)
4	8	0.993	14.92	— 2.97	3.9
4	9	0.996	15.08	— 3.03	3.9
25	5	0.994	15.16	— 3.08	3.6
25	6	0.994	16.01	— 3.49	3.6
37	6	0.998	16.94	— 4.10	3.0
37	7	0.997	17.49	— 4.34	2.8
55	1	0.972	11.05	— 1.60	7.9
55	3	0.989	15.13	— 3.33	3.6
55	4	0.994	16.03	— 3.71	3.5

Fig. 4 shows the graphic regression line of zone diameter as a function of lignosulphonic acid dilution ($\log_{10}$), incubation time 7 hrs. at 37°C. According to this, the sulphite spent liquor used contained 255 diffusion units peptide-precipitating lignosulphonic acids per 50 µl liquor. The relative uncertainty in the point of intersection between the regression line and the abcissa was found to be of the order of 3 %.

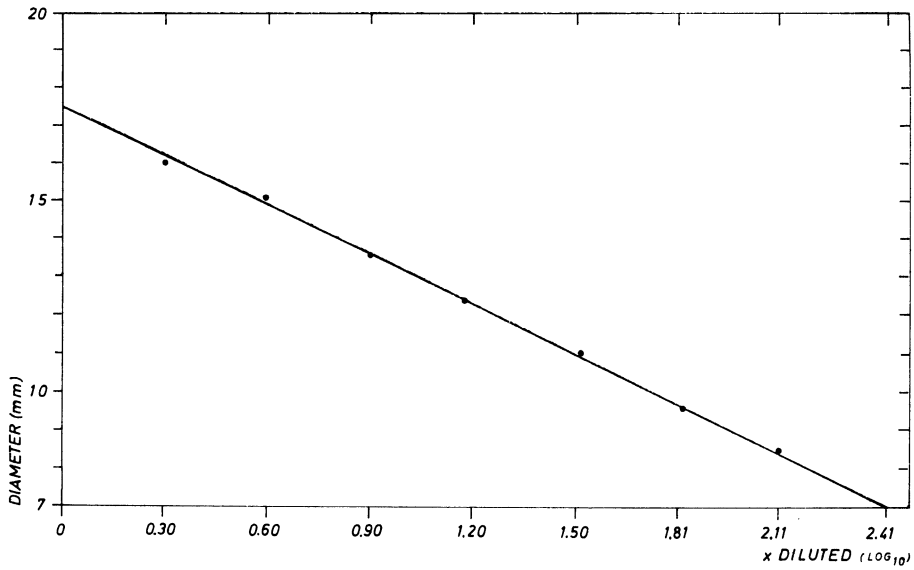

Figure 4. Semilogarithmic graphic regression line of zone diameter measured as a function of lignosulphonic acid dilution. Incubation time 7 hrs. at 37°C.

Dilution of the applied material to 1/256 did not give significant precipitation with β-naphthylamine solution, whereas 1/128 did. Using the procedure described by *Ritter* (1967), the total content of the lignosulphonic acids contained in the sulphite spent liquor was found to be 18 g/l.

DISCUSSION

As can be seen from Fig. 2, the dependence of zone diameter on the dilution of lignosulphonic acids, has exponential character. Calculations carried out with various transformations of the variables showed that $\log_{10}$ ($\times$ the dilution of the lignosulphonic acids) was a suitable transformation when the aim was to obtain straight regression lines. Table 1 shows that semilogarithmic regression lines with not significant second degree coefficients were found, for incubation at 4°C, 25°C, 37°C and 55°C, at intervals of 2 hrs. at 4°C, 25°C and 37°C. At 55°C two separate intervals with not significant second power factors were found. The relative uncertainty in the point of intersection between the abcissa and the straight regression line was found to be least for incubation 6 and 7 hrs. at 37°C. An incubation time of 7 hrs. at 37°C was chosen as suitable when 2 % neopeptone in 1.15 % agar was used for the agar precipitation procedure.

As a result of the easy and rapid application, the agar precipitation procedure may be used as a direct micro quantitative method for the determination of peptide-precipitating lignosulphonic acids in aqueous solution. The method is regarded as suitable for this purpose because of the distinctness of the precipitation zones. The procedure is considered suitable, particularly for the determination of peptide-precipitating lignosulphonic acids in sulphite spent liquors used for microbial production of proteins, and for ecological studies in fiord basins and rivers into which sulphite spent liquors are released. The proposed diffusion unit as a measure of the concentration of peptide-precipitating lignosulphonic acids refers to exactly defined conditions and is, in a modified form, in accordance with the principles of the measurement of activity due to proteolytic enzymes in casein agar plates proposed by *Sandvik* (1962). Whether all the lignosulphonic acids present in sulphite spent liquors are able to precipitate the peptides, remains to be investigated, and the results obtained cannot therefore be taken as a measure of the total ligno-

sulphonic acids present, without reservation. An important point with this procedure is that it has calculable precision, and that the measurements can be performed on a micro scale. Thus, only 50 µl was required for each sample as compared with 5 ml for the comparative method.

In addition the precipitation zones are examined without the need for any kind of developing or staining, and the method allows a great number of samples to be assayed simultaneously. The sensitivity of the method was found to be of the same order as that of the procedure based on precipitation with β-naphthylamine.

One of the problems of the agar precipitation method may be the possible variation in the thickness of the agar layer, which will affect the reproducibility of the precipitation zones. This method suffers from the same kind of disadvantages which affect other assay methods based on the principle of dilution, and care should therefore be taken to avoid long dilution series by prediluting the sample to a suitable starting concentration. In these experiments, the degree of dilution necessary to obtain end points for visible precipitation zones, did not indicate this to be a serious problem.

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SUMMARY

The effects of incubation time and incubation temperature on precipitation zones of peptide-lignosulphonic acid complexes in agar plates are studied in relation to the mathematical dependence of zone diameter as a function of the concentrations of lignosulphonic acids

in serial 2-fold dilutions. Optimal conditions are given for obtaining regression lines with not significant second degree coefficients under defined conditions.

A diffusion unit, proposed as a measure of peptide-precipitating lignosulphonic acids in aqueous solution, is defined. The possibility is discussed of using the described procedure as a micro quantitative method for the determination of peptide-precipitating lignosulphonic acids in aqueous solution.

SAMMENDRAG

Presipiteringssoner dannet av peptid-ligninsulfonsyre-komplekser i agar gel.

En direkte mikrokvantitativ metode for bestemmelse av peptidpresipiterende ligninsulfonsyrer i vandig løsning.

En har studert virkningen av inkuberingstid og inkuberingstemperatur på presipiteringssoner av peptid-ligninsulfonsyre-komplekser i agarplater i relasjon til den matematiske avhengighet av sonediameter som funksjon av konsentrasjonene av ligninsulfonsyrer i 2-fold fortynningsserie, og en har bestemt de optimale betingelser for å oppnå regresjonslinjer uten signifikante annengradsledd under definerte betingelser.

En diffusjonsenhet, foreslått som mål for peptidpresipiterende ligninsulfonsyrer i vandig løsning, er definert. Muligheten for å bruke den beskrevne framgangsmåte som mikrokvantitativ metode for bestemmelse av peptidpresipiterende ligninsulfonsyrer i vandig løsning er diskutert.

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