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GEL FILTRATION OF LIGNOSULPHONIC ACIDS AND PEPTIDE-PRECIPITATING ABILITIES OF THE SEPARATED FRACTIONS

By

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HILDRUM, KJELL IVAR and BJØRN NÆSS: *Gel filtration of lignosulphonic acids and peptide-precipitating abilities of the separated fractions.* Acta vet. scand. 1972, *13*, 293—304. — Lignosulphonic acids in dialysed sulphite spent liquor and purified lignosulphonic acids were subjected to gel chromatography on Sephadex G-75, G-100 and G-200 and the fractions tested for peptide-precipitating ability. About 56 % of the total lignosulphonic acids in the dialysed sulphite spent liquor had estimated molecular weights above 90000 and about 72 % above 44000. About 94 % of the purified lignosulphonic acids had molecular weights above 90000 and the remaining 6 % had above 36000. The major peptide-precipitating activity of the lignosulphonic acids was due to fractions with molecular weights in excess of 90000. The percentage of peptides in the peptide-lignosulphonic acid precipitates was found to be 80—90. The molecular weights of the peptides used were found to have an upper limit of about 20000. The lower limit for molecular weights of lignosulphonic acid-precipitating peptides is estimated to be below 6000.

gel filtration; lignosulphonic acids; molecular weight; peptide-precipitating ability.

During the studies on the peptide-precipitating ability of lignosulphonic acids and the effect of various proteinases on the precipitates in agar gel (*Næss* 1971a, b, c), several questions of particular interest arose concerning the details of the formation of these precipitates. Among these questions are:

1) Estimation of the distribution, in molecular size, of the lignosulphonic acids in the sulphite spent liquors.

2) Estimation of different lignosulphonic acid fractions which are capable of precipitating peptides.
3) Estimation of lignosulphonic acid fractions which can be precipitated with CaO for comparison with peptide-precipitating fractions.
4) Quantitative determination of the peptide amounts in the peptide-lignosulphonic acid precipitates.

A number of studies have been carried out in order to clarify the distribution of the molecular sizes of lignosulphonic acids in sulphite spent liquors, and to separate various lignosulphonic acid fractions. A review on these is given by *Pearl* (1967). Several other studies have been based on gel filtration (*Jensen et al.* 1962; *Brown & Falkehag* 1967; *James et al.* 1968; *Fogelberg et al.* 1968; *Gupta & McCarthy* 1968; *Forss & Stenlund* 1969). However, no reports have appeared with regard to the formation of peptide- and protein-lignosulphonic acid complexes by different lignosulphonic acid fractions.

The present work deals with the separation of lignosulphonic acids in sulphite spent liquors, according to molecular weights, and the precipitating abilities of the individual fractions.

MATERIAL AND METHODS

Sulphite spent liquors. Previous studies (*Næss* 1971a, b, c) have shown that a fermented sulphite spent liquor, from an alcohol distillery, has a comprehensive peptide- and protein-precipitating ability. This liquor which was kindly supplied by A/S Tofte Cellulosefabrik, Hurum, Norway, was, therefore, used in the present investigations. The liquor stored at —20°C was dialysed against running tap water for 15 hrs. before use.

Lignosulphonic acids. The lignosulphonic acids were prepared by precipitation of sulphite spent liquor with CaO, dissolution of the precipitate in 0.01 M-HCl, and dialysis of the solution against tap water, at room temperature, after neutralizing with 0.1 M-NaOH as described by *Næss* (1971a). The concentration of lignosulphonic acids was adjusted to 250 diffusion units of peptide-precipitating lignosulphonic acids per 50 μl liquor (*Næss* 1971b). This liquor is referred to as purified lignosulphonic acids.

Peptides and proteins. Neopeptone (Difco* 0119-01) was used for the precipitation studies. The following proteins were used for precipitation studies and for the estimation of the molecular weight distribution of the peptides in neopeptone: Insulin (Sigma**), bovine serum albumin (Sigma, mol.wt.*** 5800 and 66500, respectively) (*Mahler & Cordes* 1968) and ovalbumin (mol.wt. 45000), chymotrypsinogen (mol.wt. 25000) and ribonuclease A (mol.wt. 13700), all obtained from Pharmacia†. The isoelectric point of insulin is pH 5.35 (*Mahler & Cordes*) and of ribonuclease pH 7.8 (*White et al.* 1968).

Gel chromatography of sulphite spent liquor, lignosulphonic acids and neopeptone. The lignosulphonic acids were fractionated by passing an 0.5 ml sample through a column, 25 mm in diameter and approx. 45 cm long. The gels used were Sephadex†† G-75, G-100 and G-200. Ascending chromatography was used, and the flow rate varied between 0.40 and 0.60 ml per min., depending on the type of gel used. Water was used as eluent. Packing, stabilization and sample application were performed as described elsewhere (*Pharmacia* 1966). The absorbances of 10 ml fractions were measured at 280 nm against water using a Beckman††† DB spectrophotometer. When the absorbances exceeded 1.0, the fractions were diluted before measurement, and the values multiplied by the appropriate factors. For estimating the molecular weight distribution of neopeptone, the neopeptone and reference proteins were chromatographed on a column, as described above, using Sephadex G-75 gel. As eluent, 0.1 M borate-HCl buffer, pH 8.0, was used.

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. To 5 ml of each fraction 0.1 ml 10 % acetic acid and 0.1 ml 10 % sodium nitrite were added and the mixture shaken. After 15 min., 0.2 ml 2 M ammonia was added

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* Difco Laboratories Inc., Detroit, Michigan, USA.

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

*** Molecular weight.

† Pharmacia Fine Chemicals Inc., Piscataway, New Jersey, USA.

†† Pharmacia, Uppsala, Sweden.

††† Beckman Instruments Inc., Fullerton, California, USA.

and the mixture shaken again. After an additional 15 min. the intensity of the developed brown colour was measured at 430 nm using a Beckman DB spectrophotometer. The untreated fractions were used as blanks.

According to *Jayme & Pohl* (1967) the absorbances at 280 nm may be taken as a rough measure of the concentration of the lignosulphonic acids in sulphite spent liquor. This method was also used in this work.

Estimation of the peptide-precipitating ability of lignosulphonic acids. To 2.5 ml of each fraction, 1 ml 1 % neopeptone in 0.1 M phosphate-citrate buffer, pH 6.0, was added and the resulting turbidity measured at 600 nm. The untreated fractions were used as controls. As a measure of the precipitating ability the turbidity in relation to the amount of lignosulphonic acids present was used, $\left(\frac{\text{absorbance, 600 nm}}{\text{absorbance, 430 nm}}\right)$.

Demonstration and quantitative determination of the reactants in the peptide-lignosulphonic acid precipitate and in the supernatant. Four ml of a 5 % neopeptone solution was added to 0.5 ml of dialysed sulphite spent liquor at pH 6.0. The precipitate was centrifuged at 8000 × g and the supernatant chromatographed on Sephadex G-100 gel. The precipitate was washed once with 2 ml 0.1 M phosphate-citrate buffer, pH 6.0 and centrifuged. The precipitate was dissolved by adding a small amount of 0.02 M-NaOH and chromatographed on Sephadex G-100 gel using 0.1 M borate-HCl buffer, pH 8.0 as eluant (Fig. 3).

In order to determine the amounts of peptides and lignosulphonic acids reacting, 0.5 ml of dialysed sulphite spent liquor was added to series of 2.5—200 mg of neopeptone in 4 ml 0.1 M phosphate-citrate buffer, pH 6.0. The precipitate was centrifuged at 8000 × g and dissolved in 2 ml 0.02 M-NaOH. The protein concentration was determined in the supernatant and precipitate (Table 1) using the biuret method (*Layne* 1957). The lignosulphonic acids in the supernatant were determined by the method of *Pearl & Benson.*

RESULTS

Fig. 1 presents the results of the gel chromatography of dialysed sulphite spent liquor on Sephadex G-75, G-100 and G-200 gels. The absorbances at 280 nm of the fractions are given in

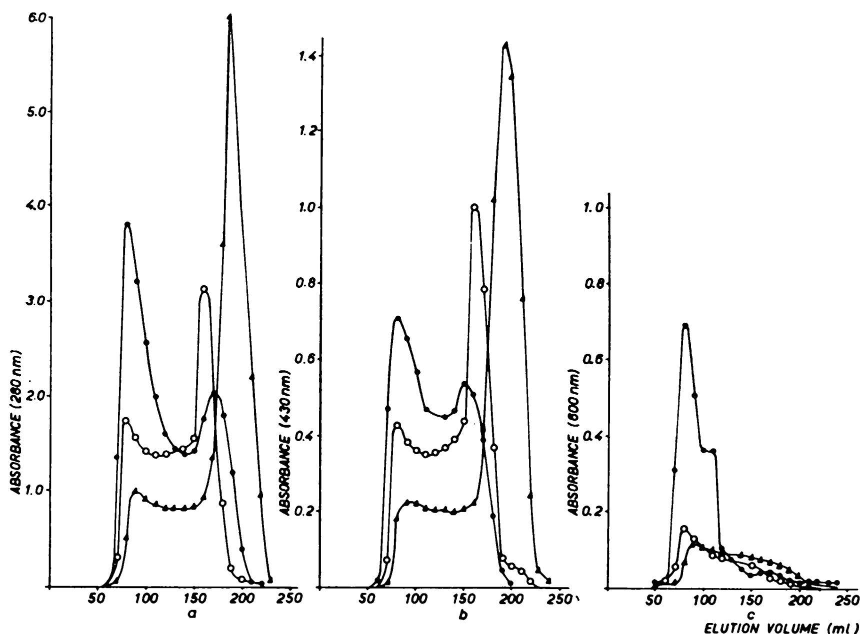

Figure 1. a: Gel chromatography of dialysed sulphite spent liquor.
b: Gel chromatography of lignosulphonic acids in dialysed sulphite spent liquor assayed according to *Pearl & Benson* (1940).
c: Gel chromatography of dialysed sulphite spent liquor and absorbance caused by the peptide-lignosulphonic acid precipitates after adding neopeptone to the fractions.
●—● Sephadex G-75, o—o Sephadex G-100,
△--△ Sephadex G-200.

Fig. 1a. On Sephadex G-75, a large peak emerges first at about the void volume of the column (Vo = 80 ml). This peak consists of molecules so large that they are almost completely excluded from the gel. As the elution volume increases, the concentration of the lignosulphonic acids declines. At elution volume, Ve = 170 ml, another peak appears, which consists of a mixture of low-molecular weight lignosulphonic acids and probably several non-ligninous substances from the sulphite spent liquor. In the Sephadex G-100 and G-200 runs, most of the high-molecular weight lignosulphonic acids are eluted in the last peak.

The procedure of *Pearl & Benson* (1940) was also used in determining the lignosulphonic acids, as other substances ab-

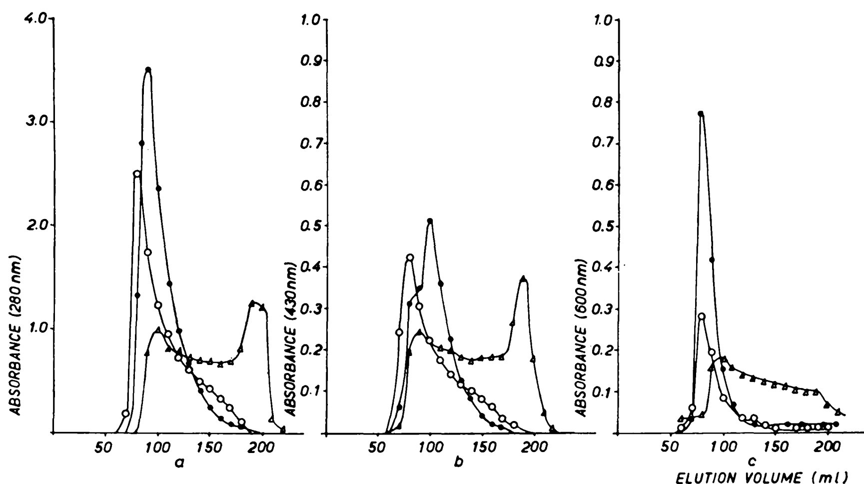

Figure 2. a: Gel chromatography of purified lignosulphonic acids.
b: Gel chromatography of lignosulphonic acids in the purified lignosulphonic acids assayed according to *Pearl & Benson* (1940).
c: Gel chromatography of purified lignosulphonic acids and absorbance caused by the peptide-lignosulphonic acid precipitates after adding neopeptone to the fractions.
●—● Sephadex G-75, o—o Sephadex G-100, △—△ Sephadex G-200.

sorbing in the u.v. region used could be present. The results of the determinations of the lignosulphonic acids in dialysed sulphite spent liquor are plotted in Fig. 1b, and it can be seen that there is good correspondance between the different methods. The turbidity measured after adding neopeptone to the fractions is shown in Fig. 1c. The amount of precipitation is variable for the different fractions.

The results of corresponding determinations as described above, using purified lignosulphonic acids are presented in Fig. 2. The purification procedure resulted in an extensive reduction in the amount of low-molecular weight substances.

Fig. 3 shows the results of the chromatography of reactants and products on Sephadex G-100 gel, after reacting neopeptone with dialysed sulphite spent liquor. The quantities of neopeptone and lignosulphonic acids reacting are shown in Table 1. The precipitate contains about 80—90 % peptides. The estimated

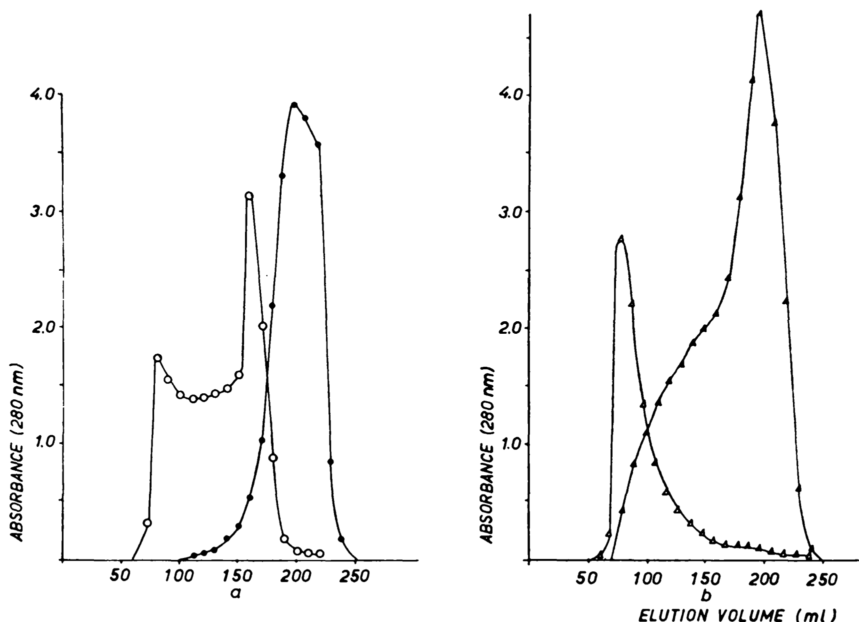

Figure 3. Gel chromatography on Sephadex G-100 of reactants (a) and products and unreacted material (b) in the formation of peptide-lignosulphonic acid precipitates.

o—o dialysed sulphite spent liquor, •—• neopeptone, △—△ dissolved peptide-lignosulphonic acid precipitate, ▲—▲ supernatant.

Table 1. Quantitative analysis of the reaction between neopeptone and the lignosulphonic acids of dialysed sulphite spent liquor in 0.1 M phosphate-citrate buffer, pH 6.0.

Peptides in		Lignosulphonic acids in		Composition of precipitate	
reaction mixture mg	precipitate mg	supernatant mg	precipitate (calculated) mg	peptides %	lignosulphonic acids (calculated) %
0	—	7.8	—	—	—
25	—	7.8	—	—	—
50	7.6	6.1	1.7	90	10
75	10.1	5.4	2.4	85	15
100	18.3	6.2	1.6	92	8
150	30.2	4.1	3.7	85	15
200	39.1	3.6	4.2	82	18

Table 2. Molecular weight distribution and peptide-precipitating ability of the lignosulphonic acids in dialysed sulphite spent liquor fractionated on Sephadex G-75 gel.

Elution volume (ml)	Elution volume / Void volume k	Eluted lignosulphonic acids (% of total)	Estimated molecular weights in each 10 ml fraction	Precipitating ability in each 10 ml fraction Absorbance, 600 nm / Absorbance, 430 nm
70	0.88	5	>90000	0.66
80	1.00	19	>90000	0.97
90	1.13	32	>90000	0.78
100	1.25	41	>90000	0.64
110	1.38	49	>90000	0.78
120	1.50	56	90000	0.23
130	1.63	61	70000	0.17
140	1.75	66	55000	0.11
150	1.88	72	44000	0.06
160	2.00	79	36000	0.08
170	2.13	86	28000	0.12
180	2.25	93	18000	0.18
190	2.38	98	10000	0
200	2.50	99	<10000	0
210	2.63	100	<10000	0

molecular weight distribution and peptide-precipitating ability of dialysed sulphite spent liquor and purified lignosulphonic acids, after fractionation on Sephadex G-75, are given in Tables 2 and 3, respectively. The estimation of the molecular weight distribution is based on the calibration curves of *Forss & Stenlund* (1969). The peptide-precipitating ability of the lignosulphonic acids is dependent on the molecular weights of the lignosulphonic acids. Gel chromatography on Sephadex G-75 of neopeptone, together with proteins of known molecular weight, showed that the neopeptone consisted of peptides with a wide range of molecular sizes. The upper limit of the molecular weights of the peptides was found to be about 20000.

Insulin, mol.wt. 5800, and ribonuclease A, mol.wt. 13700, formed precipitates with lignosulphonic acids when the pH in the reaction mixtures was adjusted to 5.0 and 7.0, respectively.

Table 3. Molecular weight distribution and peptide-precipitating ability of purified lignosulphonic acids fractionated on Sephadex G-75 gel.

Elution volume (ml)	Elution volume / Void volume k	Eluted lignosulphonic acids (% of total)	Estimated molecular weights in each 10 ml fraction	Precipitating ability in each 10 ml fraction Absorbance, 600 nm / Absorbance, 430 nm
80	0.89	12	>90000	2.47
90	1.00	44	>90000	1.17
100	1.11	66	>90000	0.30
110	1.22	79	>90000	0.19
120	1.33	88	>90000	0.13
130	1.44	94	90000	0.12
140	1.56	95	78000	0.18
150	1.67	97	64000	0
160	1.78	98	53000	0
170	1.89	99	42000	0
180	2.00	100	36000	0
190	2.11	—	30000	0
200	2.22	—	24000	0
210	2.33	—	17000	0
220	2.44	—	<7000	0

DISCUSSION

Forss & Stenlund (1969) fractionated lignosulphonic acids, according to molecular size, using gel chromatography on Sephadex G-75, and determined the molecular weights of the fractions using light scattering and sedimentation rate methods. Using their calibration curves, the distribution of molecular sizes of lignosulphonic acids in sulphite spent liquor is calculated and presented in Table 2. The calibration curves of *Forss & Stenlund,* however, cannot be used in these studies without some reservation, as *Forss & Stenlund* prepared their sulphite spent liquor from a laboratory cook from spruce chips, while the liquor used in this work was a fermented liquor obtained from a chemical wood pulp factory. The figures given, however, may be taken as good estimates. The results show that a large proportion of the lignosulphonic acids in the sulphite spent liquor has high-molecular weights. About 56 % of the total lignosulphonic acids in this preparation have molecular weights greater than 90000 and about 72 % greater than 44000. In comparison, the samples

analysed by *Forss & Stenlund* contained a considerably higher proportion of low-molecular weight lignosulphonic acids. Furthermore, *Moacanin et al.* (1955) found that the molecular weights of sodium lignosulphonates varied between 1000 and 100000, when assayed by the light scattering method. *Yean & Goring* (1964), using the sedimentation equilibrium method, obtained values between 10000 and 140000 for the molecular weights of lignosulphonates. *Gardon & Mason* (1955) found average molecular weights between 3700 and 58000 for lignosulphonates isolated from a sulphite spent liquor by dialysis, the results being obtained by osmotic measurements. However, the integral molecular weight distribution plot indicated the existence of lignosulphonates with molecular weights around 100000.

Table 2 also shows the peptide-precipitating ability of lignosulphonic acids as a function of their estimated molecular weights. The main peptide-precipitating ability is observed when the molecular weights are above about 90000. It can be seen from Table 3 that the low-molecular weight substances of sulphite spent liquor are removed by the purification procedure, while 94 % of the remaining lignosulphonic acids are estimated to have an average molecular weight over 90000. Lignosulphonic acids with molecular weights below 36000 are completely removed during the purification. The main peptide-precipitating ability is observed for molecular weights above 90000. Some of the lignosulphonic acids, possessing peptide-precipitating ability, seem to be removed during the purification procedure.

Fig. 3 indicates that the dissolved neopeptone-lignosulphonic acid precipitate contains rather small amounts of peptides. Gel chromatography of the dissolved precipitate revealed one main peak, which coincided with the high-molecular weight lignosulphonic acid peak. However, analyses of the precipitates formed by peptides and lignosulphonic acids showed that the precipitates contained 80—90 % peptides, which is in accordance with the results found by *Jantzen* (1967). The explanation is probably that after the precipitate has been dissolved, forces such as hydrogen bonds, salt bridges and electrostatic forces between the peptides and lignosulphonic acids act in such a way that the complex travels unaltered through the gel.

Insulin and ribonuclease A were found to form precipitates with lignosulphonic acids. If the molecular weight of the peptide is the only parameter determining whether the peptides are able

to form precipitates with the lignosulphonic acids when the pH in the reaction mixture is lower than their isoelectric points, the lower limit of molecular weights for lignosulphonic acid-precipitating peptides should be below 6000 (the molecular weight of insulin). On the other hand, dipeptides (mol.wt. about 200) have been found not to form precipitates with lignosulphonic acids (*Næss* 1971b). The peptides in neopeptone were found to have molecular weights below 20000.

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SAMMENDRAG

Gelfiltrering av ligninsulfonsyrer og fraksjonenes evne til å presipitere peptider.

En har separert rensede ligninsulfonsyrer og ligninsulfonsyrer i dialysert sulfittlut på Sephadex gel G-75, G-100 og G-200 og undersøkt peptidpresipiterende evne hos de ulike fraksjoner. Det ble funnet at ca. 56 % av ligninsulfonsyrene i dialysert sulfittlut hadde estimert molekylvekt over 90000 og ca. 72 % over 44000. Ca. 94 % av de rensede ligninsulfonsyrer hadde molekylvekt over 90000 og de resterende 6 % over 36000. Den største peptidpresipiterende evne ble funnet i fraksjoner med molekylvekt over 90000. En fant at innholdet av peptider i peptidligninsulfonsyrepresipitatene var 80—90 %. Peptidene som er brukt hadde molekylvekt under ca. 20000. Den nedre grense i molekylvekt for peptider som danner presipitat med ligninsulfonsyrer ble estimert til å være mindre enn 6000.

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