



OTHER SUPPORT:

GRAMINEX Flower Pollen Extract

Streptolysin Inhibitory Factor in Pollen

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The starting material used for the isolation of a factor which inhibits the hemolytic activity of streptolysin-O is a water-acetone fraction (manufacturer's name: Cernitin spissum) of a standardized pollen mixture obtained from six plant species: *Zea maize*, *Pinus montana*, *Secale cereal*, *Phleum pratense*, *Alnus glutinosa*, and *Dactylus glomerata*. The mixture is produced for commercial purposes by AB Cernelle, Ängelholm, Sweden. The inhibition of streptolysin-O by treatment with the Streptolysin Inhibitory Factor (SIF) *in vitro*, and the chemical and physio-chemical properties of the inhibiting factor have been studied.

Streptolysin-O is a heat-stable, oxygen-sensitive bacterial toxin (a metabolic product of Streptococci), belonging to a group of enzymes known as hemolysins. Under physiological conditions, streptolysin-O hemolyzes erythrocytes.

The pharmacological effect of streptolysin in Streptococcus infections has aroused considerable medical interest in the toxin.¹ Very few reports have been published concerning the inhibition of the enzymatic effect of streptolysin. No specific inhibitor of the effect of streptolysin on red blood cells is known. Inhibitory effects of fat-soluble extracts from certain plant species have been described by Kienholtz,² who found that chamomile oil from wild chamomile (*Matricaria chamomilla*) and the oil of black radish (*Cochlearia armoracia*) inhibit streptolysin activity *in vitro*. Kienholtz³ reported further, that lavender oil (*Lavendula spica*) inhibits streptolysin after a 3-day incubation period at 37°C, and that lavender oil is used medicinally in the treatment of certain bronchial disturbances.

The inactivation of streptolysin by treating with anti-streptolysin-O and trypsin has been described by Nakamura.⁴ Belgova⁵ has reported

on studies which showed that EDTA did not have any enzyme-inhibiting effect on streptolysin, whereas cobalt nitrate at a concentration of no more than 10 ppm caused strongly reduced activity. Finally, Costa *et al.*⁶ reported on studies which showed that streptolysin-O is inactivated by certain lysozymes, protamine sulphate, papain, and thymohistone.

STREPTOLYSIN INHIBITORY FACTOR

EXPERIMENTAL

1. Apparatus and equipment

The following apparatus was used in connection with gel filtration. The optical density of the eluate was measured continuously with an LKB 4701 A Uvicord I at 254.7 nm and recorded with an LKB 6520 Recorder. The fractions were collected with an LKB 7000 Ultro-Rac Fraction Collector. Spectrophotometrical measurements were carried out with a Beckman DB.

2. Purification of SIF

Fractionation with methanol. 5 ml Cernitin spissum was added slowly and under stirring to

100 ml methanol. The mixture was filtered after approx. 30 minutes shaking at room temperature. The supernatant was evaporated to approx. 3 ml by fan drying while stirring, and diluted to 5 ml with distilled water. (Cernitin spissum supernatant.) The supernatant and the sediment were tested for SIF activity.

Gel filtration on Sephadex. SIF in Cernitin spissum supernatant was purified by repeated gel filtration on a Sephadex G-15 column measuring 25 X 400 mm. The eluents used were 0.9% or 0.1% saline. After the first gel filtration run, the fractions belonging to the same UV peak were pooled and tested for SIF activity. The SIF-active fractions were concentrated to the original sample volume and gel filtrated once again. The SIF-active fractions from the second gel filtration were concentrated and re-gel filtrated under the same conditions as above. The SIF-active fractions from the third gel filtration were concentrated and analyzed.

The active fractions (the pool) from the gel filtrations were analyzed with respect to the Folin intensity (Folin-Ciocalteu reaction), and the total carbohydrate content according to Dische.⁷ The optical density of the solutions was read at 257 nm in a UV spectrophotometer.

3. Preliminary investigation of the physico-chemical properties of SIF

Heat stability. Cernitin spissum solutions, conc. 1:200, are incubated at 65°C and 100°C for 10 min. After cooling, the SIF activity of the solutions was investigated and compared with the activity of untreated SIF solution.

pH stability. The pH level of Cernitin spissum solutions, conc. 1:200, was adjusted with HCl to 2.0 or with sodium hydroxide solution to 11.5. The solutions were incubated at 37°C for 20 min, after which the pH values were adjusted to 5 (corresponding to the pH for untreated samples of Cernitin spissum). The SIF activity of the solutions was investigated and compared to the activity of untreated, incubated SIF solution.

Dialysis ability. Cernitin spissum solution, conc. 1:100, was dialyzed with the aid of a dialysis tube with a diameter of 20 mm (Union Carbide Corp.) against equal quantities of physiological saline at +4°C for 24 h. The SIF activities of the outer and inner solutions were investigated and compared with the activity of Cernitin spissum solution, conc. 1:200, stored at +4°C for 24 h, and with Cernitin spissum solution, conc. 1:200, stored in a dialysis tube at +4°C for 24 h.

Molecular weight determination. The preliminary value for the molecular weight of SIF was calculated according to Carnegie⁸ and Andrews.⁹ It was assumed that the elution volume, V_e , for SIF is approximately a linear function of the logarithm of the molecular weight. The Sephadex G-15 column, 25 x 400 mm, was calibrated with bacitracin (Mw 1422), 1 mg/ml, vitamin B₁₂ (Mw 1355), 0.1 mg/ml, erythromycin (Mw 734), the respective solutions was used for determining the elution volume.

The eluent was 0.3% saline. The V_e -values were plotted against the logarithms of the respective molecular weights, and the molecular weight for SIF was calculated from the diagram thereby obtained.

Determination of the Streptolysin Inhibitory Factor activity in Cernitin spissum. Lyophilized streptolysin-O (prepared by the "Human" Serum Research Institute, Budapest, Hungary, for diagnostic purposes) was used as the hemolyzing enzyme. The freeze-dried content of one ampoule was dissolved in 15 ml physiological saline. (According to the manufacturers, each ampoule contains 15 test-tube doses of streptolysin-O.) The substrate used was a 2% suspension of sheep blood cells in physiological saline. The concentration of blood cells in the suspension was determined by measuring the HbO₂-content of the solution in a spectrophotometer at 576 nm after dilution 1:100 with 0.05% ammonia, using a 1 cm cuvette, against the solvent as blank standard. The OD₅₇₆ for the diluted solution should be 0.182.

The streptolysin activity was investigated in a series of test samples according to the following.

To $[0.5-0.05(n-1)]$ ml streptolysin-O solution (n =dilution number) was added physiological saline to a total volume of 1.0 ml per sample. The mixtures were diluted incubated at 37°C for 30 min, after which 0.5 ml physiological phosphate buffer solution at pH 7.0, 0.2 ml physiological sodium thioglycolate solution, and 0.25 ml 2% suspension of blood cells were added to each tube. After further incubation at 37° for 30 min, the degree of hemolysis was read visually. The following gradings were used : 4 = complete hemolysis; 3 = 75% hemolysis; 2 = 50% hemolysis; 1 = 25% hemolysis; Ø = no hemolysis.

The activity of SIF in Cernitin spissum was investigated in a series of samples according to the following. To 0.5 ml of a streptolysin-O solution was added $[0.5-0.05(n-1)]$ ml of a Cernitin spissum solution (n = dilution number), diluted 1:200 with physiological saline and saline to a total volume of 1.0 ml per sample, after which the mixtures were treated and readings taken as above. The following units were used in calculating the inhibitory activity:

(1). 1.0 streptolysin-O unit (SU) = the smallest quantity of streptolysin-O, which, under the standard conditions stated above, results in complete hemolysis of blood cells (degree of hemolysis = 4). (The concentration of streptolysin units (SU) in the streptolysin solution (1 ampoule/15 ml) being approx. 8-10 SU/ml).

(2). 1.0 Streptolysin Inhibitory Factor Unit (SIF-U) = the smallest quantity of SIF, which completely inhibits 1.0 SU under the standard conditions given above.

Investigation of the inhibitory characteristics of the Streptolysin Inhibitory Factor. (1). Cernitin spissum solution, conc. 1:200, was titrated and mixed with streptolysin solution according to the standard test method. The solutions were incubated at 37°C. The incubation times for the various series of samples were: 5, 10, 15, 20, and 30 min. The degree of inhibition in the different series was investigated and compared with the inhibition of the sample that had been incubated for 30 min. (2). Streptolysin was

treated with Cernitin spissum solution containing an equivalent amount of SIF-U. The mixture was incubated at 37°C for 30 min. The streptolysin-SIF solution was dialyzed against an equal volume of physiological saline (equilibrium dialysis) in a dialysis tube of 20 mm diameter at +4°C for 24 h, after which the dialysis was allowed to continue against a large volume of saline solution for a further 24 h period at +4° C. (The volume of the outer solution was 15 times of that of the inner solution.) The SIF activity of the outer solution obtained during equilibrium dialysis was investigated and compared with the activity of untreated SIF solution that had been stored at same temperature and time. The streptolysin activity of the inner solution was investigated after 48 h dialysis and compared with the activity of untreated streptolysin that had been dialyzed under the same conditions as the treated substance, and also with streptolysin that had been stored at +4°C for 48 h.

RESULTS

Purification

Methanol fractionation. After mixing Cernitin spissum with methanol, a pale brown precipitate was obtained, having a very loose consistency. After drying, the precipitate turned dark brown. The dry weight of the precipitate was approx. 10% of the total dry weight of Cernitin spissum. The SIF activity in the extract was not reduced as a result of the methanol fractionation; all the activity was recovered in the supernatant, the sediment being completely inactive.

The OD_{257} for the supernatant was approx. 5% lower than for the untreated Cernitin spissum, the specific SIF activity $[SIF-U\ ml^{-1} [OD_{257}(conc.)]^{-1}]$ increased by about 5% (Figs. 5, 6). (The specific activity for undiluted SIF fractions was calculated with respect to the OD_{257} , the intensity of the Folin reaction and the total content of carbohydrates.)

Gel filtration. Cernitin spissum supernatant was gel filtered on analytical scale and also on preparative scale. Gel filtration on analytical scale employed a column measuring 15 X 300

mm and sample quantities of 0.2 ml. The various saline solutions used did not differ in their separative properties. The SIF activity was recovered at the elution peak with $V_e \approx 70$ ml. The fractions which gave other UV-absorption peaks were completely inactive with respect to SIF (Fig. 2).

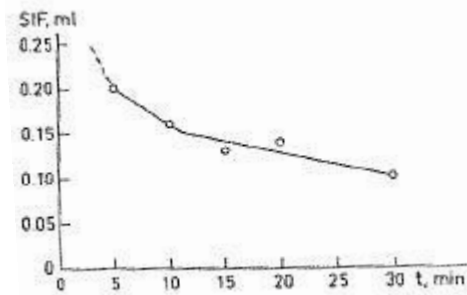


Fig. 1. Inactivation of streptolysin-O by treatment with Streptolysin Inhibitory Factor (SIF). Correlation between the quantity of SIF (ml Cernitin spissum 1:200) which inactivates 3.0 streptolysin-O units and the incubation time (t min).

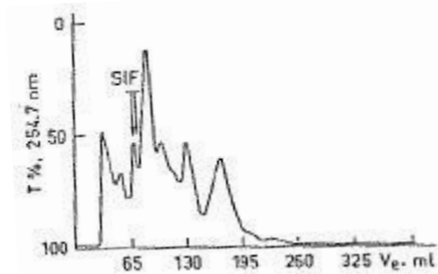


Fig. 2. Gel filtration of Cernitin spissum supernatant (1st gel filtration). Purification of Streptolysin Inhibitory Factor (SIF). Column: Sephadex G-15, 1.5x30 cm. Sample quantity: 0.2 ml. Eluent: 0.1% sodium chloride solution. Flow rate: 25 ml/h.

A number of attempts were made to concentrate the SIF active fractions: freeze-drying, vacuum drying at room temperature, and drying with cold air and hot air fans. The dried residues were then dissolved in distilled water, and the SIF activity of the solutions was investigated and compared with the activity for non-concentrated fractions. The results obtained showed that the SIF activity was completely destroyed by concentrating fractions derived from gel filtration in which 0.9% saline was used as elution fluid.

The SIF activity was not affected by drying in the fractions with a 0.1% saline concentration.

Gel filtration of Cernitin spissum supernatant was also carried out on preparative scale, using a column measuring 25x400 mm. The sample quantities were 5 ml, and a 0.1% sodium chloride solution was used as eluent. The elution curve obtained showed a high absorption for the fractions. The SIF activity was recovered after 155 ml of the elution volume, and distributed within a volume of 40 ml. (The fractions between 155 ml and 195 ml were collected.) The loss of the SIF amount introduced after the first gel filtration was approx. 25%. The second gel filtration was run on a column measuring 25x400 mm, and the sample quantity was approx. 5 ml (the concentrated pool of SIF active fractions from the first gel filtration). 0.1% saline was used as eluent.

The elution diagram showed that the sample contained a few percent high molecular impurities, but no low molecular ones, after the first gel filtration. The quantity of impurities with molecular weights slightly above and below that of SIF comprised at least half the total UV absorbing substance quantity (Fig. 3).

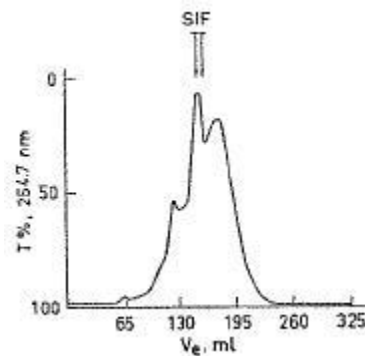


Fig. 3. Gel filtration of Cernitin spissum supernatant (2nd gel filtration). Purification of Streptolysin Inhibitory Factor (SIF). Column: Sephadex G-15, 2.5x40 cm. Sample: 5 ml concentrated pool of SIF-active fractions from 1st gel filtration (corresponding to 5 ml of Cernitin spissum supernatant). Eluent: 0.1% sodium chloride solution. Flow rate: 25 ml/h.

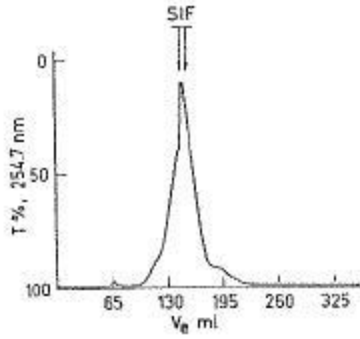


Fig. 4. Gel filtration of Cernitin spissum supernatant (3rd gel filtration). Purification of Streptolysin Inhibitory Factor (SIF). Column: Sephadex G-15, 2.5x40 cm. Sample: 3 ml concentrated pool of SIF-active fractions from 2nd gel filtration (corresponding to 5 ml of Cernitin spissum supernatant). Eluent: 0.1% sodium chloride solution. Flow rate: 25 ml/h.

The SIF activity was recovered between the elution volumes 155-195 ml. The fractions within this elution range were pooled and concentrated. In the course of the second gel filtration, a further 5% of the total SIF activity was lost.

The third gel filtration was carried out in the same way as the second. The sample volume was approx. 3 ml (concentrated pool of SIF active fractions from the second gel filtration). The elution diagram showed that the sample contained at least 5 different molecular weight groups, three of which (two with higher and one with lower molecular weight than SIF) comprised 5-7% of the total quantity of substance. The majority of the impurities, 30-40%, comprised a group with a molecular weight slightly higher than that of SIF (Fig. 4). The SIF active fractions between $V_e' = 155$ ml, and $V_e'' = 195$ ml were collected. After the third gel filtration, approx. 65% of the original SIF quantity was recovered in the active fractions.

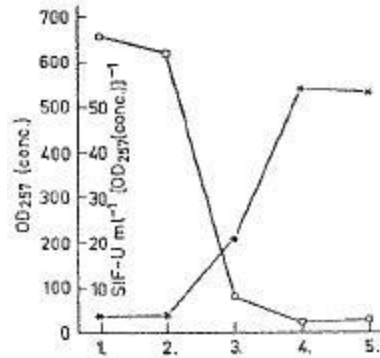


Fig. 5. Purification of Streptolysin Inhibitory Factor (SIF). Correlation between streptolysin inhibiting activity and optical density (OD_{257}). SIF-U [$OD_{257}(conc.)$] $^{-1}$: *; OD_{257} (conc.): □. Notations on x-axis: 1. Cernitin spissum. 2. Cernitin spissum supernatant from methanol fractionation. 3. SIF-active fraction from 1st gel filtration. 4. SIF-active fraction from 2nd gel filtration. 5. SIF-active fraction from 3rd gel filtration.

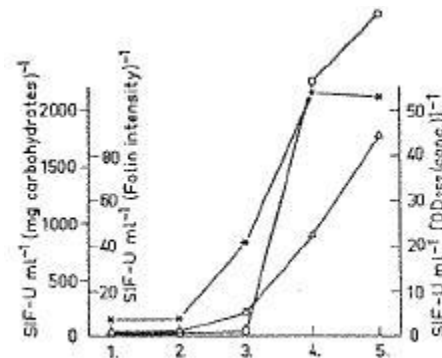


Fig. 6. Purification of Streptolysin Inhibitory Factor (SIF). Correlation between specific activity values, i.e. the streptolysin inhibiting activity in relation to the Folin intensity (SIF-U/Folin intensity: Δ), total carbohydrate content (SIF-U/carbohydrates: O), and optical density (SIF-U/ OD_{257} : *). Notations on x-axis: 1. Cernitin spissum. 2. Cernitin spissum supernatant from methanol fractionation. 3. SIF active fraction from 1st gel filtration. 4. SIF active fraction from 2nd gel filtration. 5. SIF active fraction from 3rd gel filtration.

Analyses of the SIF active fractions gave the results shown in Figs. 5 and 6. With respect to substances absorbing UV light at 257 nm, purification after the first gel filtration was 85-90%, after the second 95%, and after the third 98%.

As for purification with respect to specific SIF activity, see Table 1.

Table 1. Purification of the Streptolysin Inhibitory Factor (SIF) in pollen extract with respect to specific activity.

Degree of purity: $\frac{\text{specific SIF activity for the purified fraction}}{\text{specific SIF activity for Cernitin spissum}}$

	Degree of Purity		
	SIF-U/ml /OD ₂₅₇ conc.	SIF-U/ml/Folin Intensity	SIF-U/ml/carbohydrates
Cernitin spissum	1.0	1.0	1.0
Cernitin spissum Supernatant	1.1	1.0	1.3
1st gel filtration	5-6	4-5	2
2nd gel filtration	15	20	90
3rd gel filtration	15	40	110

Characteristics

Heat stability. SIF is resistant to heat, the activity is not reduced after incubation for 10 min at 65°C and at 100°C.

pH stability. The activity of SIF was completely destroyed after incubation for 20 min at 37°C and at a pH 2.0. At pH 11.5, approx. 60% of the SIF activity in the solution was destroyed.

Ability to dialyze. SIF is capable of dialyzing, and the activity can be quantitatively recovered in the inner and outer phases after equilibrium dialysis. The stability to dialyze is not affected by variations in the ionic strength of the solvent.

Molecular weight. The preliminary value for the molecular weight of SIF, calculated with the aid of the elution volume in gel filtration, is approx. 850. The test samples used for determining the molecular weight of SIF were Cernitin spissum supernatant and the SIF active fraction from the third gel filtration. The same results were

obtained with both samples (Fig. 7).

The SIF activity of the Cernitin spissum extract. Depending on the degree of dryness (20-40%), the SIF content in Cernitin spissum varied between 1500 and 3000 SIF-U/ml.

The dependency of the degree of inhibition on the incubation time. The test results show that, when inactivating streptolysin with SIF under standard conditions, the incubation time should not be less than 20 min (Fig. 1). An incubation time of 5 min requires a SIF concentration, which is approx. 100% greater than that required in a 30 min incubation period. The difference between 15 and 30 min is approx. 30%; this value is about 20% greater than the error of the method at the SIF concentrations used in the investigation.

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Investigation of the activity in the inner and outer dialysis phases. This investigation with SIF-treated streptolysin gave the following results. Active SIF was recovered in the outer phase in equilibrium dialysis of SIF-treated streptolysin. No reduction in activity of SIF could be observed which could be attributed to inactivation of

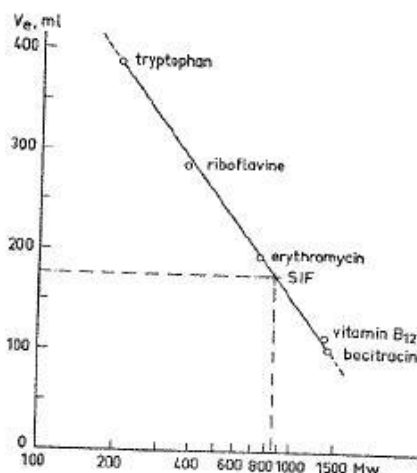


Figure 7. Determination of preliminary values for the molecular weight of the Streptolysin Inhibitory Factor (SIF), using gel filtration. Correlation between the logarithm for the molecular weight and the elution volume (V_e). Column: Sephadex G-15, 2.5X40 cm. Eluent: 0.3% sodium chloride solution.

streptolysin. The SIF activity in the outer phase was somewhat less than half of the original SIF activity in the inner phase inside the dialysis tube, but this halving of the concentration agreed with the dilution and was fully comparable with the results obtained in equilibrium dialysis of SIF without streptolysin. The activity of the streptolysin in the inner phase was investigated after continued dialysis against a large volume of outer phase. The results of the test showed that no streptolysin activity could be recovered after inactivation with SIF. The activity of the dialyzed streptolysin that had not been treated with SIF, diminished after 48 h dialysis by about 20% in relation to non-dialyzed streptolysin.

Storage stability. The SIF activity for Cernitin spissum solution, conc. 1:200, does not diminish during storage at room temperature or at +4°C over a period of several days. The stability of the solution is limited primarily by fungal infection, even at low temperatures. The stability of concentrated and diluted, sterile Cernitin spissum solutions is unlimited at refrigeration temperature, with respect to the SIF activity. (The streptolysin solution can be stored at +4°C for at least 48 h without suffering any loss of activity. At higher temperatures, the solution easily becomes infected. Careful handling in the presence of air does not lead to a reduction in activity.)

DISCUSSION

The Streptolysin Inhibitory Factor, SIF, is a quantitatively measurable substance occurring in pollen extract. The method described for the measurement of SIF activity is reproducible. The error of the method at lower SIF concentrations is approx. 10%, but somewhat lower at higher concentrations. The investigation had shown that SIF activity is not affected by smaller variations in incubation time or in temperature. The inhibitory activity of SIF is a linear function of the SIF concentration within a wide range.

The molecular weight of ~840 indicates that SIF is probably a condensation of a polymerization

product. Other possible alternatives might be a peptide, a nucleic acid fragment, or a polysaccharide. SIF is easily soluble in water, in saline, and in 95% methanol. The fact that SIF is soluble in methanol does not eliminate any of these proposed groups of substances, since the dielectric constant of the solvent is high.

The heat stability and the stability against variations in the ionic strength of the solvent indicate a chemical structure in which the streptolysin-inhibiting activity is either reversible or resistant to these factors. The SIF activity is destroyed in association with treatment in acid environment, and partly also in alkaline environment. In view of the fact that the acid-alkali treatment takes place under mild conditions, it is hardly feasible that a component, e.g. acid or alkali sensitive amino acids, in SIF would thereby be destroyed.

The results obtained from the gel filtrations show that the SIF activity is probably not dependent on a co-factor. Whereas the inhibitory effect of SIF remains unchanged after its reaction with streptolysin, the latter is irreversibly inhibited, as shown by the fact that SIF can be separated from inactivated streptolysin by dialysis. The chemical analyses show that the specific SIF activity, with respect to the Folin intensity and the total carbohydrate content, increased strongly after the third gel filtration in relation to the second filtration. Concerning the optical density at 257 nm, the specific activity of the SIF active fractions was unchanged after the third gel filtration in comparison with the second filtration. This result increased the probability, that SIF might be a nucleic acid fragment.

The results of separate investigations have shown that pollen extract, produced under sterile conditions, contains a concentration of SIF which is just as high as that of the commercially produced Cernitin spissum extract.

Further studies on isolation and characterization of SIF are in progress.

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