9. T. F. Solov'eva, I. M. Ermak, O. D. Bondarenko, G. M. Frolova, and Yu. S. Ovodov, Microbios, 25, 133 (1979).
10. I. M. Ermak, T. F. Sokol'eva, O. P. Sanina, and Yu. S. Ovodov, in: IVth All-Union Biochemical Congress. Abstracts of Scientific Communications [in Russian], Vol. 2 (1979), p. 181.
11. T. G. Vasil'eva, G. I. Sazonets, and N. I. Shapiro, Dokl. Akad. Nauk SSSR, 211, 711 (1973).
12. D. C. Morrison and L. Lieve, J. Biol. Chem., 250, 2911 (1975).
13. V. E. Eskin, Light Scattering by Solutions of Polymers [in Russian], Moscow (1973), p. 46.
14. B. G. Belen'kii and L. Z. Vilenchik, The Chromatography of Polymers [in Russian], Moscow (1978), p. 189.

QUANTITATIVE ANALYSIS OF β-EXOTOXIN IN INSECTICIDAL PREPARATIONS BY HIGH-PERFORMANCE ANION-EXCHANGE CHROMATOGRAPHY

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A quantitative method is proposed for determining β-exotoxin, which is a product of the vital activity of certain serotypes of *Bac. thuringiensis*. The method is based on the use of high-performance anion-exchange chromatography. The chromatography of β-exotoxin-containing preparations was performed on a column (1.6 × 150 mm) containing type 2632 anion-exchange resin (Hitachi) in a concentration gradient of HCl and NaCl. The time of analysis amounted to 34 min. The method permits the analysis of β-exotoxin in insecticidal preparations in an amount of 1 μg and upwards.

The heat-stable β-exotoxin present in complex bacterial insecticidal preparations obtained from *Bac. thuringiensis* is 2-O-[4'-O-(adenosin-5''-yl)-ξ-D-glucopyranosyl]-4-phosphoallaric acid. In 1976, Prystaš et al. [1] effected the complete synthesis of this compound, opening up prospects for its industrial production. At the present time, a number of biological and biochemical tests based on its action on insects [2-8] and also on the investigation of its inhibiting influence on the DNA-dependent RNA-polymerase from *E. coli* and *Bac. thuringiensis* have been developed for the analysis of preparations of β-exotoxin. However, these methods are complex and require considerable expenditures of time. We have proposed a method for the quantitative determination of β-exotoxin in various insecticidal preparations which is based on the use of high-performance anion-exchange column chromatography.

As the stationary phase we selected type 2632 spherical anion-exchange resin (Hitachi), which is based on a copolymer of styrene and divinylbenzene. The use of an anion-exchange resin is connected with the presence in the β-exotoxin molecule of a phosphomonoester group capable of dissociation in aqueous solutions with the formation of anions. Being distinguished by a high capacity, polymerization ion-exchange resins permit the separation of complex multicomponent systems without the necessity, in the majority of cases, for the preliminary purification of the samples being analyzed [11, 12]. This is of great importance, since as a rule, the β-exotoxin preparations contain a considerable amount of inorganic salts, protein compounds, carbohydrates, and other impurities.

As the mobile phase we used aqueous solutions of NaCl and HCl. To achieve the best resolution of the components of the mixtures being analyzed and the separation of the β-exotoxin peak we tried various forms of gradient curves permitting both the concentration of the salt and the pH of the eluent to be changed during the separation process.

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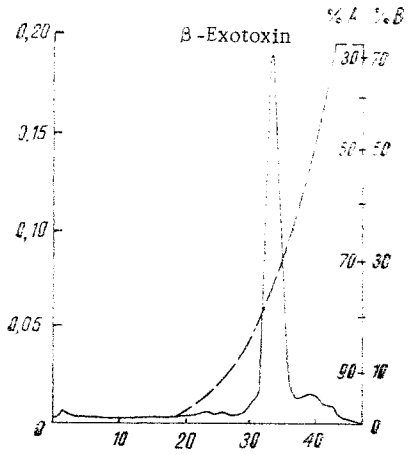

Fig. 1

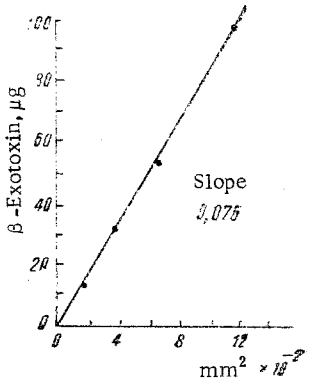

Fig. 2

Fig. 1. Chromatogram of a standard β-exotoxin preparation on a column (1.6 × 150 mm) of polymerization anion-exchange resin, type 2632 (Hitachi) in a gradient of HCl and NaCl concentrations at 50°C. Solution A — 0.025 N HCl and 0.05 M NaCl; B — 0.01 N HCl and 0.2 M NaCl. Rate of elution, 18 ml/h. The full line is the absorption of the eluate at 260 nm, and the dashed line the change in the proportion of solution B in the mixing chamber.

Fig. 2. Calibration graph of the dependence of the area of the peak of β-exotoxin on its concentration in the sample deposited on the column.

Figure 1 shows a chromatogram of a purified standard solution of a β-exotoxin preparation and shows the optimum form of the gradient ensuring the complete separation of the UV-absorbing impurities present in insecticidal preparations from the peak corresponding to the phosphorylated form of β-exotoxin. In order to accelerate analysis, chromatography was performed at 50°C.

The detection of the compounds in the eluate was performed by the spectrophotometric method at a wavelength of 260 nm which, according to Bond et al. [2], corresponds to the absorption maximum of the β-exotoxin. The identification of the β-exotoxin peak on the chromatogram was done by comparing the retention time of the substance with the corresponding characteristics of a standard preparation. In addition, samples were analyzed in parallel by thin-layer chromatography on Silufol UV-254 plates (Czechoslovakia) and the biological activities of the main components isolated from the initial preparations in the process of chromatography were determined. The retention time of the β-exotoxin on the column under the conditions given was 34 min. The time of an analysis can be shortened by a further optimization of the separation parameters (in particularly, by using a wider and shorter column permitting a considerable increase in the rate of elution of the compounds).

For the quantitative analysis of β-exotoxin in unknown preparations, a calibration curve of the dependence of the area of the peak of this substance on its concentration in the sample deposited on the column was plotted. Figure 2 shows that at concentrations of β-exotoxin of from 1 to 100 μg in the volume of sample deposited the calibration curve is linear; the calibration factor amounts to 0.76 μg/mm^2. The sensitivity of the method permits β-exotoxin to be determined when its amount in a sample is 1 μg and more. Under these conditions the standard error is less than ±4%.

Figures 3 and 4 present typical chromatograms of some preparations of insecticide and of the culture liquid obtained in the fermentation of *Bac. thuringiensis* var. *insectus* in the All-Union Scientific-Research Institute of Microbiological Plant-Protecting Agents and Bacterial Preparations. The dephosphorylated form of the β-exotoxin is eluted immediately after the dead volume of the column, and the lactone, in agreement with the results of Sebesta et al. [13, 14], after the β-exotoxin peak. The percentages of β-exotoxin in the samples mentioned were determined on the initial weight of the substance taken for analysis.

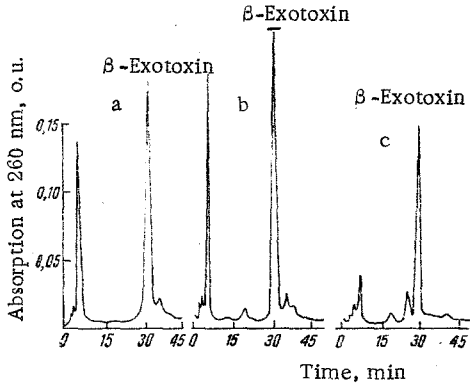

Fig. 3

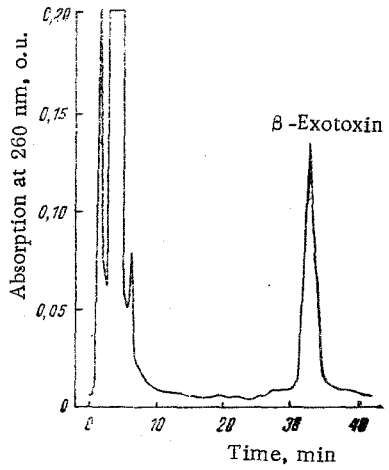

Fig. 4

Fig. 3. Chromatograms of three β-exotoxin-containing preparations. Amounts of β-exotoxin in the preparations: a) 25%; b) 36%; c) 50%.

Fig. 4. Chromatogram of a culture liquid obtained from the industrial fermentation of *Bac. thuringiensis* var. *insectus* containing 13% of β-exotoxin.

The results that we obtained agree well with those of analyses performed with the aid of thin-layer chromatography.

The use of high-performance anion-exchange chromatography permits the analysis of β-exotoxin-containing preparations without the preliminary preparation and special purification of the samples. The method is characterized by high accuracy and productivity. Columns with the polymerization anion-exchange resin possess good reproducibility and a long working life (several months) under conditions of constant use. The method for the quantitative determination of β-exotoxin described in the present paper is recommended for the performance of routine analyses in the production of this insecticide.

EXPERIMENTAL

The investigation was carried out with chemical reagents having the analytical degree of purity, and with deionized water for the preparation of the chromatographic systems. The standard sample of β-exotoxin and β-exotoxin-containing insecticidal preparation, and also of culture liquids, were obtained by the procedure described previously in the All-Union Scientific-Research Institute of Microbial Plant-Protecting Agents and Bacterial Preparations.

The thin-layer chromatography of the β-exotoxin-containing preparations was performed on Silufol UV-254 plates (Cavalier, Czechoslovakia). The mobile phase used was n-propanol—NH_4OH (conc.)—H_2O (10:2:5, v/v). The substances absorbing UV light were detected on the chromatograms with the aid of a Minuvis chromatoscope (USA) at a wavelength of 254 nm. The R_f value of β-exotoxin in this system was 0.21.

The analysis of the biological activities of the components present in the insecticidal preparations and the culture liquid was carried out on the test insect *Musca domestica* in the All-Union Scientific-Research Institute of Veterinary Medicine by a procedure described previously [16].

Column Chromatography. Column anion-exchange chromatography was performed in a model 635T high-pressure liquid chromatograph (Hitachi), fitted with a spectrophotometer with flow-through cells having a volume of 8 μl and with a device for creating a gradient. A thermostated steel column (1.6 × 150 mm) filled with type 2362 spherical polymerization anion-exchange resin (Hitachi) was used. The resin was first washed repeatedly with 1 N NaOH with water, with 1 N HCl, and again with water to neutrality. The column was filled by the method of Scott and Lee [17] after the careful degassing of the resin suspension.

For gradient elution we used the following degassed solutions: A — 0.0025 N HCl and 0.005 M NaCl; B — 0.01 N HCl and 0.02 M NaCl. The rate of elution was 18 ml/h. The working temperature of the column was 50°C. The form of the gradient curve used is shown in Fig. 1. The β-exotoxin in the eluate was detected at a wavelength of 260 nm.

After each analysis the column was regenerated by being washed successively with solutions B and A for 10 min. To check the calibration curve, aliquots of a standard solution of β-exotoxin were chromatographed and the areas of the peaks were determined from the formula

$$S = \frac{H \cdot W_{H/2}}{2},$$

where H is the height of the peak, S is the area of the peak, and $W_{H/2}$ is the width of the peak at half-height.

The calibration factor, or the slope of the calibration curve in the region of its linearity, was 0.076 μg/mm². The standard error in the quantitative analysis of the β-exotoxin in no case exceeded ±4%.

SUMMARY

1. A high-performance chromatographic method of determining β-exotoxin in bacterial insecticide preparations is proposed which permits the analysis of this compound in nanomolar amounts.

2. The amounts of β-exotoxin in a number of insecticidal preparations and a culture liquid have been determined.

LITERATURE CITED

1. M. Prystaš, L. Kalvoda, and F. Šorm, Collect. Czech. Commun., 41, 1426 (1976).
2. R. P. M. Bond, C. B. C. Boyce, and S. J. French, Biochem. J. 114, 477 (1969).
3. A. Burjerjon and H. Barlac, Ser. Entomophage, 2, 221 (1964).
4. J. Vankova, Acta Bogemoslov, 63, 10 (1966).
5. E. Schmidt and G. Benz, Experientia, 25, 96 (1969).
6. A. Krieg, Ent. App., 9, 185 (1966).
7. G. Benz and J. M. Perron, Experientia, 8, 871 (1967).
8. G. E. Cantwell, A. M. Heimpel, and M. J. Tompson, Insect, 6, 466 (1964).
9. G. Rosenberg, G. Carlberg, and H. G. Gyllenberg, J. Appl. Bact., 34, 417 (1971).
10. J. Carlberg, Rep. Dept. Microbiol. Univ. Helsinki, 6, 90 (1973).
11. P. R. Brown, High Pressure Liquid Chromatography: Biochemical and Biomedical Applications, Academic Press, New York (1973).
12. V. K. Kagramanova, S. N. Bubenshikova, and L. A. Baratova, Usp. Khim., 48, 957 (1969).
13. K. Sebesta, K. Horska, and J. Vankova, Insect Pathology and Microbial Control, North-Holland, Amsterdam (1967), p. 238.
14. K. Sebesta, K. Horska, and J. Vankova, Collect. Czech. Commun., 34, 891 (1969).
15. V. B. Freiman, O. A. Aleshina, N. K. Fedorov, S. B. Tikhomirova, L. P. Sinitsina, and N. A. Ostrogskaya, Tr. VNII Bakpreparat., No. 1, 50 (1973).
16. A. P. Tonkonozhenko and M. B. Kats, Tr. VNIIVS, 63, 9 (1979).
17. C. D. Scott and N. E. Lee, J. Chromatogr., 42, 263 (1969).