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The Purification of Subtilin Concentrates by Counter-current Distribution

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The production and purification of the antibiotic subtilin, and methods of assay, have been described.[1] A recent paper[2] has presented improvements in the purification procedure and evidence for the homogeneity of the product, based on electrophoretic studies and constancy of properties after fractionation by precipitation and hydrolysis. The present paper describes the fractionation of subtilin concentrates by the counter-current distribution technique.[3] Some characterizing properties of the purified subtilin are recorded.

Subtilin has been obtained only in amorphous form and concentrates of widely differing microbiological activities cannot be distinguished by the more common physical criteria or analytical measurements. It seemed desirable to apply to the concentrates a method of purification which could simultaneously afford evidence of the purity of the products and, hence, a study of the counter-current distribution of the material was undertaken.

Experimental

A. Butanol-Water Distribution.—The distributions were carried out in separatory funnels, using mutually saturated butanol and water as the two phases. After shaking the funnels, the resulting emulsions were separated, by centrifugation if necessary, and lower (aqueous) phases were transferred. Fractions were numbered starting with 1. At the completion of a distribution, the organic layer in each funnel was diluted with two volumes of petroleum ether and, after shaking, the aqueous phase was removed. The organic mixture was then extracted with one-fourth its volume of water, and this aqueous solution added to the original water layer. About 90% of the concentrate was usually recovered in this way.

The total amount of material isolated from a given fraction was determined from the optical density of the aqueous extract at 2775 Å. and at 2850 Å., or by actual weight of lyophilized product, or by both. Good agreement between the two methods was generally obtained. The spectrophotometric determination of total material in solution was based on the observation that the ultraviolet absorption spectra of subtilin concentrates of widely differing activities were almost identical (see below).

A 2.5-g. sample of a subtilin concentrate[4] having an activity of 24,000 u./mg.[5] was put through a 13-plate counter-current distribution, using 200 ml. of each solvent phase per funnel. The results are summarized in Table I.

Fractions 12 and 13 (1.0 g.) from the first distribution were put through an 11-plate separation, using 100 ml. of each phase per fraction. The results are shown in Table II.

Fractions 10 and 11 (0.5 g.) from the second distribution were subjected to a 20-plate process, using 100 ml. of each phase per funnel. Table III contains the results of this distribution, and also the calculated weights for each fraction, based on a partitition coefficient of 0.170.

TABLE I

FIRST DISTRIBUTION

Fraction	Weight, mg.	Activity, u./mg.
1	10	....
2	27	....
3	25	....
4	26	....
5	17	....
6	18	....
7	16	....
8	30	....
9	54	17,000
10	116	20,000
11	331	25,000
12	514	25,000
13	527	27,000
14	158	21,000

TABLE II

FIRST DISTRIBUTION

Fraction	Weight, mg.	Activity, u./mg.
1	1	....
2	1	....
3	1	....
4	1	....
5	2	....
6	2	....
7	14	....
8	54	22,000
9	151	....[a]
10	282	26,000
11	220	26,000
12	44	19,000

[a] Fraction lost during lyophilization.

TABLE III

THIRD DISTRIBUTION

Fraction	Wt., obsd., mg.	Wt., calcd., mg.	Activity, u./mg.
1	0	....	....
2	.6	....	....
3	.2	....	....
4	1.1	....	....
5	0.1	....	....
6	.8	....	....
7	.5	....	....
8	1.5	....	....
9	0.5	....	....
10	1.4	0.01	....
11	1.7	.07	....
12	2.2	.4	....
13	3.9	1.8	....
14	8.5	6.5	....
15	19.0	19.2	23,000
16	42.3	45.2	26,000
17	74.2	83.2	27,000
18	115.2	115.2	26,000
19	110.3	112.8	27,000
20	61.3	69.8	24,000
21	2.2	20.5	....

(1) Lewis, Feeney, Garibaldi, Michener, Hirschmann, Traufler, Langlykke, Lightbody, Stubbs and Humfeld, *Arch. Biochem.*, **14**, 415 (1947); Stubbs, Feeney, Feustel, Lightbody and Garibaldi, *ibid.*, **14**, 427 (1947); Lewis, Humphreys, Thompson, Dimick, Benedict, Langlykke and Lightbody, *ibid.*, **14**, 437 (1947); Dimick, Alderton, Lewis, Lightbody and Fevold, *ibid.*, **15**, 1 (1947); Hassall, *Nature*, **161**, 137 (1948).

(2) Fevold, Dimick and Klose, *Arch. Biochem.*, **18**, 27 (1948).

(3) Craig, *J. Biol. Chem.*, **150**, 33 (1943); **115**, 519 (1944); Craig, Golumbic, Mighton and Titus, *ibid.*, **161**, 321 (1945); Hogeboom and Craig, *ibid.*, **162**, 363 (1946); Williamson and Craig, *ibid.*, **168**, 687 (1947).

(4) The authors are indebted to Dr. James C. Lewis of the Western Regional Research Laboratories, Albany, California, for this sample.

(5) Tube dilution units for *Micrococcus conglomeratus*.

Counter-current distribution of less pure subtilin concentrates also resulted in a large increase in activity. In a typical example, a 504-mg. portion of 11,000 u./mg. material was put through a 12-plate distribution, using 50 ml. of each solvent per fraction. End fractions yielded poor material (1000 to 11,000 u./mg.), but fractions 9, 10 and 11 gave 54 mg., 17,000 u./mg.; 61 mg., 18,000 u./mg.; and 76 mg., 18,000 u./mg., respectively.

B. *s*-Butyl Alcohol-Water Distribution.—A 300-mg. portion of material of 26,000–27,000 u./mg. activity, derived from a peak fraction of a distribution with butanol and water, was put through a 23-plate distribution in which *s*-butyl alcohol was substituted for butanol.[6] The observed yields per fraction for the last ten fractions, together with those calculated for a partition coefficient of 0.185, are presented in Table IV. No material was found in fractions 1 through 14.

TABLE IV

s-BUTYL ALCOHOL–WATER DISTRIBUTION

Fraction	Wt., obsd., mg.	Wt., calcd., mg.
15	0.4	1.0
16	2.5	3.3
17	7.5	9.1
18	18.1	20.1
19	35.2	36.2
20	51.8	52.0
21	55.5	55.5
22	40.3	43.0
23	12.3	21.1
24	2.6	5.0

C. Properties of Purified Subtilin. Analytical.—For fraction 19 of Table III, dried in weighing pigs for 15 hours at 25°, found: C, 49.83, 49.61; H, 6.71, 6.95; N, 14.93; S, 5.44. For fraction 20 of Table IV, dried in weighing pigs for 2 hours at 56°, found: C, 51.05; H, 7.10; N, 15.17; S, 4.95; amino-N, 1.5%. Titration with lithium hydroxide gave an equivalent weight of 3340.

A sample with an assay value of 19,000 u./mg. showed, when dried in weighing pigs for 4 hours at 25°, the composition: C, 51.53, 51.76; H, 6.89, 6.50; N, 15.54, 15.65; S, 4.60, 5.05; methoxyl, none.

Polarographically, in 0.1 *M* lithium hydroxide solution at a concentration of 2.3 mg./ml., subtilin showed no evidence of reducible material.

Ultraviolet Absorption Spectra.—In aqueous solution, the ultraviolet absorption spectra of subtilin samples of quite different microbiological activity were very similar. The curves were characterized by no maxima, but did possess a shoulder at 2750–2800 Å., and an inflection point at 2850 Å. Table V presents the $E^{1\%}_{1cm.}$ values for some typical subtilin samples.

TABLE V

ULTRAVIOLET ABSORPTION DATA

Activity, u./mg.	$E^{1\%}_{1cm.}$ at 2775 Å.	$E^{1\%}_{1cm.}$ at 2850 Å.
700	13.5	12.1
9,000	18.3	14.6
18,000	17.8	14.5
24,000	16.8	13.8
26,000	16.6	13.6

Infrared Spectrum.—A sample of thrice-distributed subtilin mulled in petrolatum showed in the infrared a distinctly peptide-like spectrum with the following bands: strong at 3.10, 6.04, 6.50 and 6.58 μ; medium at 7.79 and 8.09 μ; and weak at 8.65 and 9.66 μ.

(6) Dr. R. L. Peck of these Laboratories first demonstrated the practicability of using *s*-butyl alcohol for the counter-current distribution of subtilin.

Optical Activity.—A highly purified and carefully dried subtilin sample showed a specific rotation of $[\alpha]^{25}D$ −36 ± 1° (*c*, 0.865 in water).

Discussion

The conditions under which the counter-current distributions of subtilin were carried out have not been ideal. For the efficient purification of an impure material, a distribution coefficient near unity is desirable,[3] but this was not achieved here. Due to the necessity of centrifuging to break the emulsions which were frequently encountered, small mechanical losses and imperfections in the transfer of the phases occurred. Also, it was necessary to displace as much subtilin as possible from the organic to the aqueous phase by addition of petroleum ether before determining the amount of material present, an obviously less than quantitative process.

In view of the necessary limitations of the process as applied here, it is interesting to note the agreement between calculated and observed distributions, as shown in Tables III and IV. Within the limits of error of the assay, the microbiological activity of the subtilin reached a value (26,000–27,000 u./mg.) after the first counter-current distribution which did not change on repeated distribution or when the organic phase was changed from normal to secondary butyl alcohol. The analyses of preparations from the two distributions (Tables III and IV) indicated constancy of composition.

Examination of the microbiological activities of the fractions isolated from the consecutive counter-current distributions (see Tables I to III) of subtilin reveals a trend to lower activities on either side of the peak fraction, although this trend does diminish with each distribution and lies within the experimental error in the last experiment. A possible explanation is that some inactivation occurs in solution during the distribution process. If this is the case, the ideal goal of a distribution yielding all fractions of equal activity, but theoretical distribution by weight, could never be attained.

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Summary

Subtilin concentrates have been subjected to counter-current distribution processes using *n*-butyl alcohol and water and *s*-butyl alcohol and water. The products so obtained appear to be essentially homogeneous. Some physical measurements and analytical data on subtilin purified in this manner are presented.

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