PREPARATION AND PROPERTIES OF SUBTILIN

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In recent years it has been shown that α,β-unsaturated acid residues are present in many natural substances. In view of this, the hypothesis has been put forward that compounds of this series are important intermediates in the biosynthesis of some unusual fragments of peptides of microbial origin. It is assumed, for example, that the epimerization of L-amino acids into the D forms and the formation of α-hydroxyamino acids takes place via dehydroamino acids; it is also considered that dehydroamino acids are nothing other than precursors of a number of linear systems (lanthionines) and cyclic systems (thiazolidine, thiazole, oxazole, etc.). Interesting examples of such transformations are given by Bycroft [1].

In addition to this, it is known that residues of α,β-unsaturated amino acids can impart specific functional or physiological activity to peptides or proteins. Thus, dehydroalanine is a component of the active center of histidine ammonia lyase [2] and, possibly, of phenylalanine ammonia lyase [3]. So far as concerns the antibiotics nisin and subtilin which we have studied, their antibacterial activity is due to the presence of ε-dehydroalanyllysine as the C-terminal fragment [4, 5]. It must also be noted that the comparatively high content of unsaturated amino acids (in nisin 3 out of 29; in subtilin not less than 2 out of 28) and sulfur-containing amino acids (7 and 5, respectively) imparts a series of unusual physicochemical properties to these compounds. For example, nisin is capable of polymerizing in aqueous solution, giving polymers with molecular weights of up to 35,000 [6]. It is sensitive to the action of oxidizing agents and undergoes degradation in a weakly alkaline medium with β-elimination in respect of the lanthionine residues. Thus, even at the isolation stage the danger arises of obtaining preparations which are heterogeneous, both because of polymerization and because of the modification of individual fragments. We have previously reported the preparation of nisin under conditions practically excluding its inactivation. The isolation scheme

TABLE 1. Amino Acid Composition of Subtilin

Amino acids	No. of residues, time of hydrolysis, h				Literature data [9, 10]
	24	48	72	96	
Lysine	3,10	2,03	3,30	2,30	3
Aspartic acid	1,05	1,05	1,09	1,01	1
Glutamic acid	3,40	3,06	3,28	3,03	3
Glycine	2,06	2,02	1,94	1,93	2
Alanine	1 10	1,02	1,04	0,99	1
Valine	0,37	0,62	0,75	0,75	1
Isoleucine	0,94	1,15	1,07	1,01	1
Leucine	4,00	3,93	4,06	4,05	4
Phenylalanine	0,93	0,96	0,90	0,98	1
Lanthionine	3,10	4,05	3,74	4,85	1
Methyllanthionine	1,20	0,91	1,06	0,77	4
Proline	+	2,02	+	2,36	1
Sarcosine	+	0,88	+	1,76	2
Tryptophan*	?	?	?	?	1

*The content of tryptophan was not determined.

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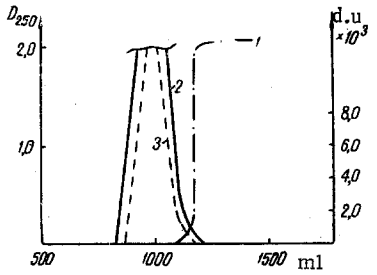

Fig. 1

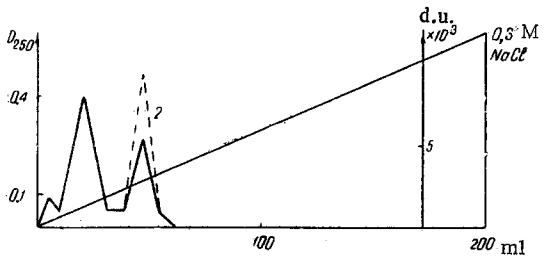

Fig. 2

Fig. 1. Gel filtration of a concentrate of subtilin through Sephadex G-25 [column 90 × 5.6 cm, eluent 0.2 M acetic acid, charge 150 ml of solution with an activity of 0.3 γ/ml (Sarcina lutea) rate 250 ml/h]: 1) electrical conductivity; 2) optical density at 250 nm; 3) dilution units.

Fig. 2. Analytical chromatography of subtilin on CM-cellulose (column 40 × 0.9 cm, eluent 0.01 M sodium acetate buffer, pH 4.8, sodium chloride concentration gradient, charge 30-40 mg of a preparation with an activity of 0.03 γ/ml): 1) optical density at 250 nm; 2) dilution units.

included gel filtration and chromatography on CM-cellulose [7]. As we have shown, the same operations may be useful in the purification of other antibiotics related to nisin. In the present paper we describe a method for the isolation of subtilin and give its properties.

A series of methods has been proposed previously for the isolation of subtilin, but only chromatography on silica gel and countercurrent distribution bave been used with success [8-10]. By just these methods, American workers have obtained a preparation of subtilin A characterized by its amino acid composition (Table 1). As we have shown previously, countercurrent distribution leads to considerable inactivation of the antibiotics of the nisin group. The scheme which we propose enables chromatographically homogeneous subtilin with a high antibacterial activity to be obtained. It follows from Table 1 that the amino acid composition found agrees well with literature data. Only some deviations in the content of proline and an inversion of the contents of lanthionine and β-methyllanthionine are observed. We did not determine the content of tryptophan and α,β-unsaturated amino acids.

EXPERIMENTAL

As the producing agent for subtilin we used the strain Bacillus subtilis ATCC 6633; its properties and the conditions of fermentation have been described by Feeney et al. [11]. The B. subtilis was grown in flasks with shaking for 72 h on a medium containing, in addition to mineral salts, sucrose (100 g/liter), citric acid (2 g/liter), glutamic acid (2 g/liter), and L-asparagine (2 g/liter). At the end of fermentation, the pH of the culture fluid (15 liters) was brought to 5.5. The antibiotic was extracted in the cold with 7.5 liters of butanol. Sodium chloride (100 g/liter) was added to the extract, and it was kept in the cold for several hours. The precipitate that had deposited was separated in a centrifuge and dried in a vacuum desiccator. The concentrate (2 g) was dissolved in 0.5 N acetic acid (2 × 400 ml), the solution was evaporated at 30°C to 150 ml, and the precipitate was filtered off with suction. The resulting solution was desalted by gel filtration through Sephadex G-25 (Fig. 1). The fractions containing the antibiotic were freeze-dried. A preparation (237 mg) with an activity of 0.015 γ/ml (Sarcina lutea) was chromatographed on CM-cellulose (0.01 M sodium acetate buffer, pH 4.8) in a sodium chloride concentration gradient (Fig. 2). The fractions containing the antibiotic were combined and concentrated at 30°C. The sodium chloride was eliminated by gel filtration under the conditions described above. After freeze-drying, 42 mg of a colorless amorphous preparation with an activity of 0.002 γ/ml with respect to Sarcina lutea (the activity was determined by the serial-dilution method with a bacterial load of 1000 cells/ml and incubation at 37°C for 19 h) and 0.07 γ/ml with respect to Staph. aureus was obtained.

The preparation gave a single spot on chromatography in a thin layer of MN 300 cellulose in the amyl alcohol–isopropanol/butanol–pyridine–water (1:1:1:3:3) system [12]. When the N-terminal amino acids were determined in the form of the DNS (dansyl) derivatives, only ε-DNS-lysine was found in the hydrolysate:

$[\alpha]_D^{25}$ −36° (c 0.5; H_2O). Literature data: $[\alpha]_D^{26}$ −34.4° (c 0.2; 1% acetic acid [8]), $\varepsilon_{250\,nm}$ 7000 (at mol. wt. 3500); 4.5 mg of subtilin was hydrolyzed with 4 ml of 5.7 N HCl at 105 ± 2°C for 24, 48, 72, and 96 h. The amino acid composition was determined on a Unikhrom analyzer. The results of the analysis are given in Table 1.

SUMMARY

A simple method for obtaining subtilin has been proposed which enables a homogeneous preparation with a high antibacterial activity to be isolated.

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