THE NUMBER AND NATURE OF α,β-UNSATURATED AMINO ACIDS IN SUBTILIN

Erhard Gross and Hans Hermann Kiltz

Section on Molecular Structure
Laboratory of Biomedical Sciences
National Institute of Child Health and Human Development
National Institutes of Health
Bethesda, Maryland 20014

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SUMMARY: In subtilin, a peptide produced by Bacillus subtilis, there are present three α,β-unsaturated amino acids, namely, two residues of dehydroalanine and one residue of β-methyldehydroalanine (dehydrobutyrine). Subtilin and nisin, a polypeptide produced by Streptococcus lactis, thus have in common not only the COOH-terminal sequence dehydroalanyllysine but also the number and nature of α,β-unsaturated amino acids.

INTRODUCTION: The peptide antibiotics nisin (1) and subtilin (2) are identical in the COOH-terminal sequence dehydroalanyllysine (1, 2). Nisin contains three α,β-unsaturated amino acids, two residues of dehydroalanine and one residue of β-methyldehydroalanine (3).

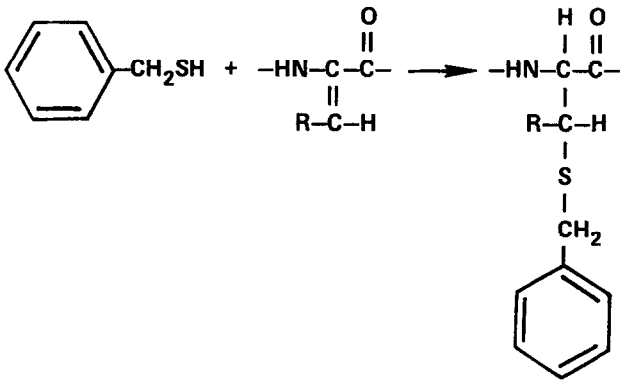
Following methylmercaptoacetate addition, the amino acid analysis of subtilin suggested the presence of more than one residue of α,β-unsaturated amino acids (2) in the molecule. This observation and other close relationships between nisin and subtilin, such as molecular size (2) and identical numbers of residues of lanthionine and β-methyllanthionine, prompted a study of the total number of α,β-unsaturated amino acids in subtilin.

α,β-Unsaturated amino acids present were expected to be revealed by the addition of benzylmercaptan to the double bond according to equation 1 and the formation of S-benzylcysteine and/or β-substituted S-benzylcysteine.

METHODS and RESULTS: Subtilin was purified by gel chromatography on Sephadex G-25 and by countercurrent distribution (4).

S-Benzylcysteine was purchased from Aldrich Chemical Co., Inc., Milwaukee, Wisconsin, and twice recrystallized from ethanol:water = 1:1.

β-Methyl-S-benzylcysteine was synthesized according to the method of



(1)

R = H: dehydroalanine (formation of S-benzylcysteine)
R = CH_3: β-methyldehydroalanine (formation of β-methyl-S-benzylcysteine)

Carter et al. (5).

S-Benzylcysteine and the diastereoisomers of β-methyl-S-benzyl-cysteine are readily resolved in a single column system (60 cm) of the amino acid analyzer using a third buffer (1.2 N in Na^+; pH 5.20). Under these conditions, they are eluted between ammonia and arginine (Figure 1).

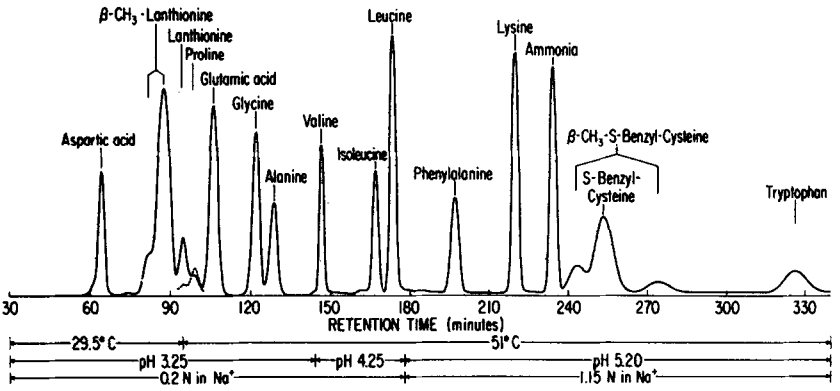


Fig. 1.: Amino acid analysis of subtilin after benzylmercaptan addition.

The addition of benzylmercaptan to the α,β-unsaturated amino acids was investigated in three different ways: (a) on subtilin, (b) on subtilin after treatment with hydrogen chloride in glacial acetic acid, and (c)

on a fragment of subtilin obtained by cleavage with trypsin.

(a) Subtilin was dissolved in N-ethylmorpholine-acetate buffer of pH 8.5, which contained 50% ethanol, and a 200-fold excess of benzylmercaptan was added. The reaction vessel was evacuated and flushed several times with nitrogen before it was sealed under vacuum. The mixture was allowed to react for 14 days at room temperature after which time it was lyophilized and passed over a Sephadex G-25 column (6 x 120 cm, 0.2 N acetic acid). The amino acid analysis of the addition product showed the presence of 0.95 residues of β-methyl-S-benzylcysteine and 1.75 residues of S-benzylcysteine (Figure 1).

(b) Subtilin was treated with hydrochloric acid in glacial acetic acid at 110° C for 10 min (2). Benzylmercaptan was added to the lyophilized product under the conditions described above. After passage over a Sephadex G-25 column (6 x 120 cm, 0.2 N acetic acid), the amino acid analysis of this material showed 0.60 residues of β-methyl-S-benzylcysteine but no S-benzylcysteine.

(c) Treatment of subtilin with trypsin results in the cleavage of both lysyl peptide bonds in the molecule. A dipeptide, H_2N-Try-Lys-COOH (T_1), is removed from the H_2N-terminal region, and a tripeptide, H_2N-ILEU-DHA-LYS-COOH (T_3), from the COOH-terminal region of the molecule:

H_2N–TRY–LYS–LAN----------LYS–ILEU–DHA–LYS–COOH

$\leftarrow T_1 \rightarrow \leftarrow T_2 \rightarrow \leftarrow T_3 \rightarrow$

Benzylmercaptan was allowed to react with the tryptic fragment T_2, and the addition product was passed over a Sephadex G-25 column (6 x 120 cm, 0.2 N acetic acid). The amino acid analysis of the addition product showed the presence of 0.99 residues of S-benzylcysteine and 0.95 residues of β-methyl-S-benzylcysteine (Figure 2).

The numbers of residues of lanthionine and β-methyllanthionine of the three addition products were identical with those of the starting materials.

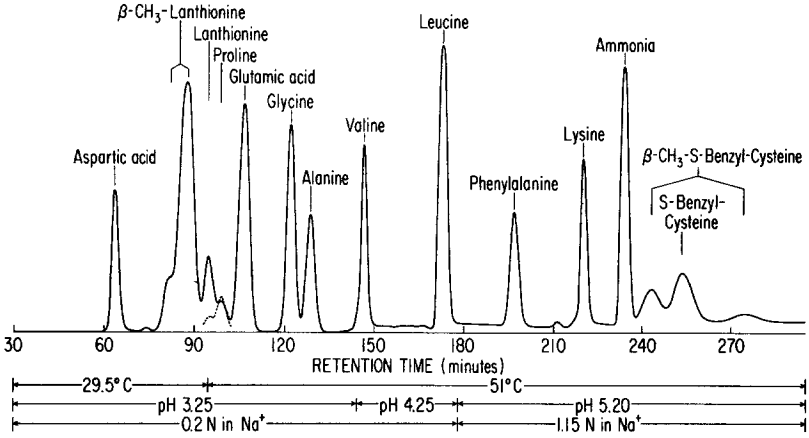
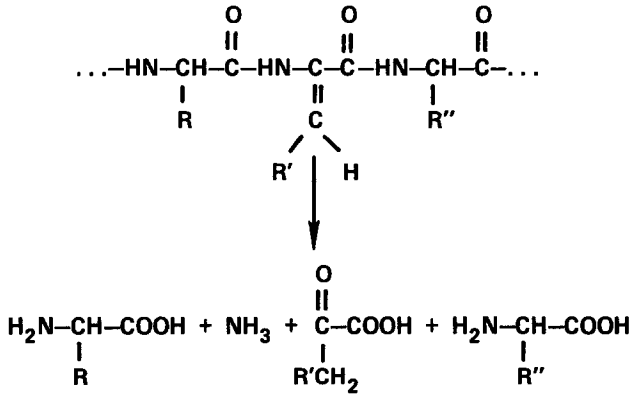


Fig. 2.: Amino acid analysis of the tryptic peptide T_2 of subtilin after benzylmercaptan addition.

Differences in the numbers of moles of ammonia before and after mercaptan addition (Table 1) are due to the degradation of the α,β-unsaturated amino acids upon total hydrolysis of the starting materials and the formation of ammonia and α-keto acids (Equation 2).



(2)

The UV-absorption of the α,β-unsaturated amino acids at 250 mμ is abolished upon the addition of mercaptan, and the molar extinction coefficient (ϵ_{250}) is greatly reduced (Table 1).

DISCUSSION: The appearance of two residues of S-benzylcysteine and one residue of β-methyl-S-benzylcysteine in the amino acid analysis of subtilin that was treated with benzylmercaptan, establishes the presence of

TABLE 1

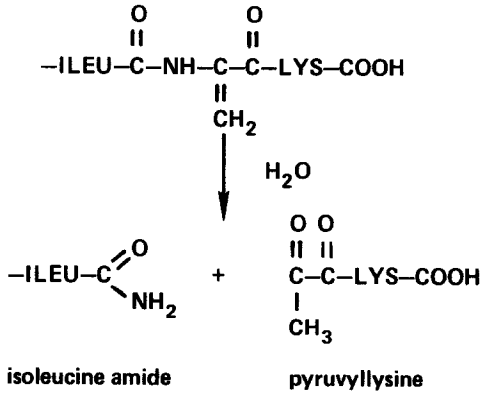
Moles of ammonia and molar extinction coefficients of subtilin and its tryptic peptide T_2 before and after mercaptan addition

	Ammonia		ϵ_{250}*	
	Before mercaptan addition	After mercaptan addition	Before mercaptan addition	After mercaptan addition
Subtilin	5.9	3.2	8400	1300+
Fragment T_2	5.1	3.3	6200	1560+

*After subtraction of the absorption due to tryptophan.
+The molar extinction coefficient of S-benzylcysteine at 250 mμ is 450.

two residues of DEHYDROALANINE and one residue of DEHYDROBUTYRINE in the molecule.

Treatment of subtilin with hydrogen chloride in glacial acetic acid degrades the dehydroalanine residues with the formation of amide and pyruvic acid (cf. Equation 3, formulated for the dehydroalanine residue in the penultimate position of subtilin, where the reaction results in the formation of pyruvyllysine)



(3)

Dehydrobutyrine is stable to the conditions employed here, and one residue of β-methyl-S-benzylcysteine is found in the amino acid analysis

of the addition product of benzylmercaptan to subtilin that was treated with hydrogen chloride in glacial acetic acid.

The presence of one residue each of S-benzylcysteine and β-methyl-S-benzylcysteine in the amino acid analysis of the addition product of benzylmercaptan to the tryptic peptide T_2 places dehydrobutyrine and the second residue of dehydroalanine into endopositions of this fragment.

The lower number of moles of ammonia, seen in the amino acid analyses of the addition products, is in agreement with the conversion of the respective numbers of α,β-unsaturated amino acids to thioether amino acids. Of the four monaminodicarboxylic acids of subtilin, three have been reported to be present as amides (4). The amino acid analysis of subtilin records 6 moles of ammonia, that of its benzylmercaptan addition product 3.2 moles. The tryptic peptide T_2 contains the four monaminodicarboxylic acids of subtilin. The amino acid analyses of this fragment before and after benzylmercaptan addition record 5.1 and 3.3 moles of ammonia, respectively.

Correcting for the presence of one residue of tryptophan, a molar extinction coefficient ϵ_{250} of 8400 was calculated for subtilin. This approximates a contribution of 3000 per residue of α,β-unsaturated amino acid (3). The extinction coefficient of the tryptic peptide T_2 was determined to be 6200. Following benzylmercaptan addition, the extinction coefficients decreased to 1300 and 1560, respectively. The remaining absorption is due to (a) the newly formed benzylthioethers and (b) possibly a small amount of unreacted dehydroalanine and dehydrobutyrine.

The presence of two residues of dehydroalanine and one residue of dehydrobutyrine further extends the relationships between subtilin and nisin (2; 6). Although the two peptides are produced by rather distantly related micro-organisms, thus far it has been established that they have in common (a) number and nature of α,β-unsaturated amino acids (3), (b) numbers of lanthionine and β-methyllanthionine residues (4;6), and (c) the COOH-

terminal sequence DEHYDROALANYLLYSINE (2).

While as yet not conclusively established, vital biological functions are attributed to α,β-unsaturated amino acids. The prediction made earlier (3), that the presence of α,β-unsaturated amino acids is not restricted to peptides of relatively low molecular weight of microbial origin, has been substantiated. Dehydroalanine has been reported to be present in L-phenylalanine ammonia lyase of potato tubers (7) and in L-histidine ammonia lyase of Pseudomonas putida (8;9).

The chemical interactions between α,β-unsaturated amino acids, on the one hand, and amides and α-keto acids, on the other hand, have been pointed out repeatedly (6). It is interesting to observe that in Pseudomonas putida there is also present an enzyme, namely urocanase (10), which utilizes covalently bound α-ketobytyrate as prosthetic group. It would not come as a surprise to see this keto acid related to dehydrobutyrine.

Finally, a first report has appeared identifying dehydroalanine in a mammalian system, namely, histidine ammonia lyase of rat liver (11).

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