TRYPTIC CLEAVAGE OF ENZYME A IN BACITRACIN SYNTHETASE

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1. Introduction

The enzyme complex bacitracin synthetase which is composed of the three multifunctional enzymes A, B and C [1] possesses at least 53 catalytical functions (reviewed in [2]). The enzyme complex is represented in fig.1.

In the present studies the presence of separate catalytic domains in enzyme A has been investigated. When trypsin (0.05–0.5 μg/ml) was added to purified enzyme A (mol. wt 335 000) a partial cleavage of the enzyme in smaller subunits was observed. The molecular weights of the smallest subunits, which still were able to activate the single amino acids L-isoleucine, L-cysteine, L-leucine and L-valine, were estimated to be ~72 000.

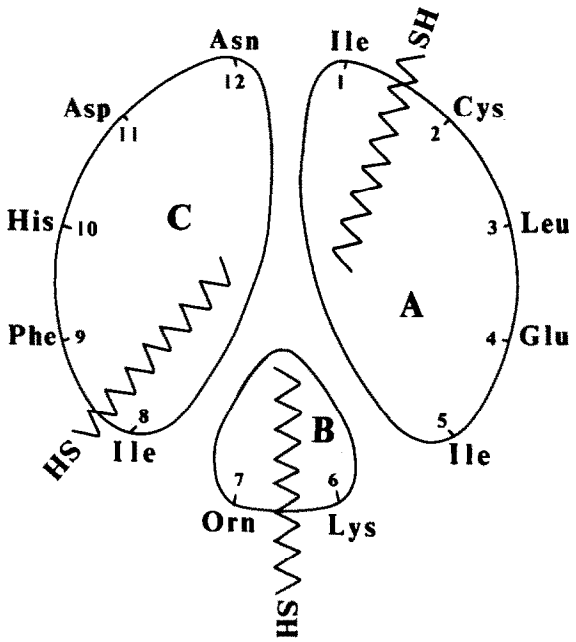

Fig.1. Schematic representation of bacitracin synthetase. A, B and C represent the 3 complementary enzymes of bacitracin synthetase. The activation sites of the constituent amino acids (numbered 1–12 from the N-terminal end) in bacitracin, are indicated on the figure. The zig-zag line illustrates covalently linked phosphopantetheine.

2. Methods and materials

2.1. *Growth of* Bacillus licheniformis

B. licheniformis ATCC 10716 was grown as in [3].

2.2. *Fractionation of bacitracin synthetase*

The 43–49% satd. $(NH_4)_2SO_4$ fraction [4] was prepared and enzyme A was fractionated and purified by Ultrogel ACA 34 gel filtration and Bio-Gel HPT hydroxyapatite chromatography as in [5].

2.3. *Trypsin treatment of enzyme A*

Enzyme A was precipitated after hydroxyapatite chromatography by the addition of solid $(NH_4)_2SO_4$ to saturation. Bovine serum albumin (0.02%) was added as a carrier protein. Enzyme A was solubilized in buffer A (0.04 M potassium phosphate, 0.002 M $MgSO_4$, pH 7.2). Trypsin (type III, 11 900 BAEE units/mg protein, Sigma Chem. Co, St Louis) treatment was carried out at 35°C for 30 min as in section 3.

2.4. *Estimation of ATP–$^{32}PP_i$ exchange reaction*

The incubations were carried out at 37°C for 30 min and the exchange reactions were determined according to [4].

2.5. *Estimation of bacitracin synthesis*

The incubations were carried out at 37°C for 30 min as in [3] and the amount of bacitracin synthesis was determined [4] using thin-layer chromatography and radioautography.

2.6. *Enzymes of gramicidin S synthetase*

The light and the heavy enzymes of gramicidin *S* synthetase were kindly provided by K. Aarstad, Department of Biochemistry, University of Oslo.

2.7. *Labelled substances and measurement of radioactivity*

L-[U-^{14}C]isoleucine and $Na_4{}^{32}P_2O_7$ were obtained from New England Nuclear, Boston, and from The Radiochemical Centre, Amersham. For counting a Packard Tri-Carb Liquid Scintillation counter and a Frieseke and Hoepfner gas-flow counter were used.

2.8. *Calculation of the distribution coefficient*

The distribution coefficient (K_{av}) is determined from the relation:

$$K_{av} = \frac{V_e - V_o}{V_t - V_o}$$

where V_e is the elution volume of the protein, V_o is the void volume and V_t is the total volume of the gel bed.

3. Results

3.1. *Tryptic inactivation of bacitracin synthetase*

Trypsin treatment (0.4–1.0 μg trypsin/ml) of purified enzyme A of bacitracin synthetase resulted in 50% reduction of the activation activities for the amino acids L-isoleucine, L-cysteine and L-leucine as shown in table 1. The result for L-valine is indicated in the table since this amino acid may substitute for L-isoleucine in the activation reaction [6]. The result for L-glutamic acid is not shown due to the low level of the ATP–$^{32}PP_i$ exchange reaction for this amino acid [4].

By using the enzymes B and C in the activation reactions for the other amino acids of bacitracin, similar results were obtained (results not shown). However, at this trypsin concentration (0.4–1.0 μg/ml) 85–90% reduction of the complete bacitracin formation was observed when unfractionated bacitracin synthetase was tested.

Table 1
Tryptic inactivation of enzyme A

Amino acid addition	The trypsin concentrations (μg/ml) which reduces the activity 50%
Isoleucine	1.0
Cysteine	0.5
Leucine	0.4
Valine	1.0

Enzyme A from 20 g wet cells [4] was solubilized in 8 ml buffer A. Fractions (1 ml) were treated with 0.1–2.0 μg trypsin and the ATP–$^{32}PP_i$ exchange reactions for the amino acids L-isoleucine, L-cysteine, L-leucine and L-valine were measured (results not shown). The trypsin concentrations which reduce 50% of the activation reaction for the individual amino acids are indicated in the table

3.2. *Tryptic cleavage of enzyme A of bacitracin synthetase*

Trypsin treatment (0.2 μg trypsin/ml) followed by gel filtration chromatography on Ultrogel ACA 34 demonstrates a partial cleavage of enzyme A into smaller fragments as seen in fig.2. The molecular weights of the peaks 1–4, fig.2, were determined to be 335 000, 200 000, 145 000 and 72 000, respectively (see fig.3). Peak 1 represents enzyme A, while peak 4 seems to represent the smallest activation units for the individual amino acids which are normally activated by enzyme A. Peaks 2 and 3 probably represent enzyme A minus 2 and 3 activation units. These peaks are most likely intermediate cleavage products of enzyme A. No peak representing enzyme A minus 1 activation unit could be detected. This might be due to the kinetics of the tryptic cleavage of enzyme A. These experiments were carried out with enzyme A after Ultrogel fractionation and after hydroxyapatite chromatography.

Less trypsin (0.05 μg trypsin/ml) resulted in a reduction of peaks 2 and 3. In this experiment peak 4 could not be seen. More trypsin (0.5 μg/ml) resulted in an accumulation of peak 3. However, the level of peak 4 was almost the same as in fig.2. This might be due to instabilities of the smallest subunits. The possibility that peaks 2 and 3 could contain aggregates of the smallest activation units is under investigation.

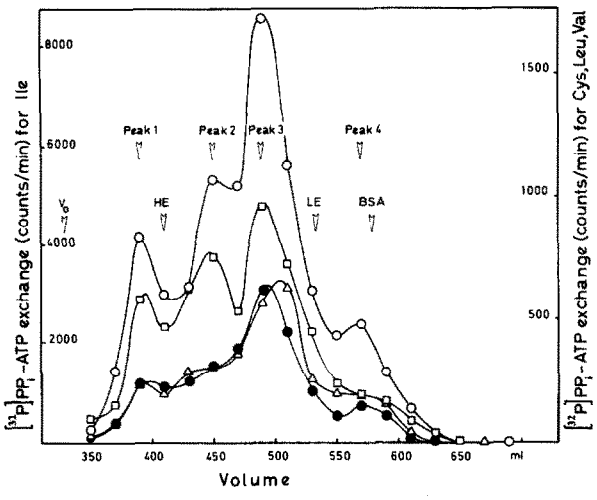

Fig.2. Fractionation of trypsin treated enzyme A on Ultrogel ACA 34. Enzyme A (after Ultrogel ACA 34 gel filtration) from 20 g wet cells was solubilized in 8 ml buffer A, treated with trypsin (0.2 μg/ml) and fractionated on an Ultrogel ACA 34 column (3.2 × 100 cm) at 4°C. The column was eluted with 0.05 M potassium phosphate buffer (pH 7.4) containing 0.2 mM $MgCl_2$ and 10% glycerol (15 ml/h). The ATP–$^{32}PP_i$ exchange reactions for the amino acids L-isoleucine (○), L-cysteine (□), L-leucine (△) and L-valine (●) were estimated in 0.2 ml aliquots of each elution fraction (10 ml). Total amounts of radioactivity in each incubation mixture were 302 000 cpm. The radioactivity measured in control tubes (50–60 cpm) without any amino acid addition, was subtracted. The exchange activity for isoleucine has been reduced by a factor of 5 for convenience. The positions for bovine serum albumin (BSA) and the light (LE) and the heavy enzymes (HE) of gramicidin *S* synthetase are indicated on the figure. The positions of LE and HE were estimated by measuring the ATP-$^{32}PP_i$ exchange reactions for D-phenylalanine and L-proline, respectively [2].

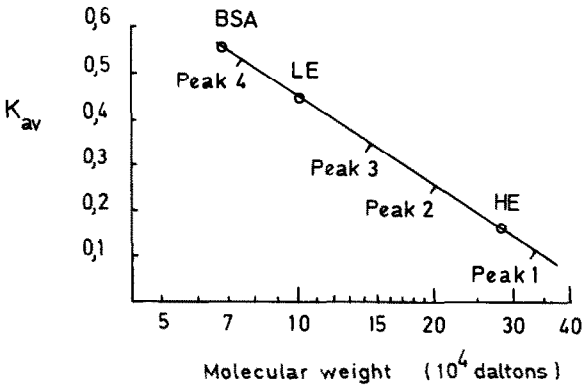

Fig.3. Estimation of the molecular weights of the tryptic products of enzyme A. The distribution coefficient (K_{av}) for the peaks 1–4 from fig.2 were calculated. The linear correlation between K_{av} for globular proteins and the log molecular weights were used for the molecular weight estimations. As reference substances BSA (68 000 daltons), LE (100 000 daltons) and HE (280 000 daltons) were used.

4. Discussion

The 3 multifunctional enzymes A, B and C of bacitracin synthetase occur simultaneously in the growth phase of *Bacillus licheniformis* [3]. Not much is known about the origin and structure of this procaryote enzyme complex. The present investigations indicate that enzyme A consists of separate domains, one for each amino acid which it normally activates. The domains which might be defined as distinct structural compact regions which could be completely encircled by a closed surface [7], are probably held together by open peptide bridges. Since they are cleaved by trypsin, the bridges seem to contain at least one arginine or one lysine residue.

The centers for the amino acid activation seem to be located on the domains and not between the domains as reported for other multifunctional enzymes [8]. However, the elongation steps in bacitracin synthesis have to take place as interdomain reactions. The different levels for trypsin inactivation of the catalytical sites of bacitracin synthetase probably reflect the amino acid composition or the three dimensional structure around the active centers of the enzyme.

In bacitracin synthetase other types of domains might also be present. For instance, presence of a smaller protein, split off by a factor in the fermentation broth, which acts as a carrier of the phosphopantetheine arm in tyrocidine synthetase, was reported [9].

The origin of the domains has not been investigated in this work. The folding of the protein chain into domains may occur simultaneously as a cooperative process. Separate domains could also be formed at an earlier stage of growth before being linked together.

The mild tryptic cleavage of enzyme A provides a method for further studies on the inter- and intradomain reaction of peptide synthetases.

Acknowledgements

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