

SYNTHESIS OF BEAUVERICIN BY A MULTIFUNCTIONAL ENZYME

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Beauvericin synthetase, a multifunctional enzyme catalyzing depsipeptide formation in *Beauveria bassiana* was purified to near homogeneity. The enzyme consists of a single polypeptide chain with a molecular mass of about 250 kdaltons. The mechanism of beauvericin formation is very similar to that of the cyclohexadepsipeptide enniatin.

The constituents of the beauvericin molecule, L-phenylalanine and D- α -hydroxyisovaleric acid are activated as thioesters *via* the corresponding adenylates. N-Methylation takes place at the thioester bound stage of the phenylalanine residues. Omission of the methyl donor S-adenosyl-L-methionine results in the formation of demethylbeauvericin.

Studies on substrate specificity revealed that phenylalanine could be replaced by a number of other aromatic or aliphatic amino acids like β -phenylserine, *ortho*-, *meta*-, *para*-fluorophenylalanine, isoleucine, norleucine and leucine. Valine, the constituent amino acid of enniatin B was not accepted by the enzyme.

Beauvericin is an insecticidal cyclohexadepsipeptide antibiotic with ionophoretic properties produced by different *Fungi Imperfecti* like *Beauveria bassiana*¹⁾, *Paecilomyces fumosoroseus*²⁾ and the plant pathogenic fungus *Polyporus sulphureus*³⁾, a basidiomycete. It has an enniatin-like structure and is composed of three residues of D- α -hydroxyisovaleryl-N-methyl-L-phenylalanine which are linked by ester bonds (see Fig. 1).

The enniatins are synthesized by a multifunctional enzyme consisting of one polypeptide chain with a molecular mass of about 250 kdaltons. The precursors of the enniatin molecule are activated as thioesters and covalently bound substrate amino acids are N-methylated. Then peptide bond formation and cyclization reactions occur^{4,5)}.

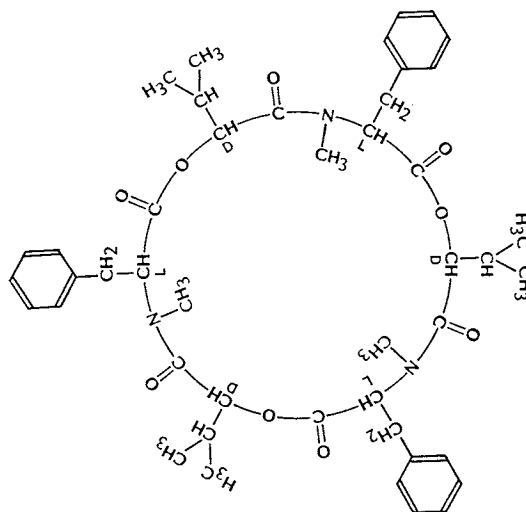
In the case of beauvericin we have recently shown by crude extract studies that this depsipeptide is also synthesized by a soluble enzyme system from D- α -hydroxyisovaleric acid (D-HYIV) and phenylalanine (L-Phe) under consumption of ATP and S-adenosyl-L-methionine (AdoMet)⁶⁾.

The present paper describes the purification and characterization of the multifunctional enzyme beauvericin synthetase from *B. bassiana* which represents another multifunctional enzyme system of the enniatin synthetase type.

Materials and MethodsChemicals and Radioisotopes

Radioactive phenylalanine (specific activity: 510 Ci/mol), S-adenosyl-L-[methyl-¹⁴C]methionine

Fig. 1. Structure of beauvericin.



(specific activity: 57 Ci/mol) and D,L-[1-¹⁴C]valine (specific activity: 60 Ci/mol) were purchased from Amersham. Radioactive D,L-HYIV was prepared from D,L-valine by deamination with HNO₂⁷⁾. Na₄-[³²P]pyrophosphate (2.9 Ci/mol) was from Du Pont-New England.

Beauvericin, D-HYIV and *N*-methyl-L-phenylalanine (NMePhe) were obtained from Bachem (Bubendorf, Switzerland).

2-Amino-4-methylhex-4-enoic acid was a gift from Dr. L. FOWDEN (London, GB).

All other reagents and solvents were of highest purity commercially available.

Organism and Growth Conditions

B. bassiana (Bals.) Vuill. was grown for 50~55 hours in 3% molasses, 1% corn steep liquor as described previously⁶⁾. Beauvericin titer was checked by the picric acid assay as described by AUDHYA and RUSSEL⁸⁾. The mycelium was harvested by suction filtration and frozen at -80°C after washing with 0.5 M KCl.

Enzyme Preparation

Preparation of crude extracts from lyophilized mycelium, poly(ethyleneimine) precipitation and fractional ammonium sulfate precipitation were carried out as described previously⁶⁾. For all steps, except the sucrose gradient centrifugation, 50 mM potassium phosphate buffer (pH 7.2), containing 4 mM dithioerythritol (DTE) and 0.25 mM EDTA (buffer A) was used.

Assay of Beauvericin Synthetase

This was measured by incorporation of [¹⁴C]Phe into beauvericin as described previously⁶⁾.

Gel Filtration on Ultrogel Aca 34

The ammonium sulfate pellet collected between 25~35% saturation was dissolved in a minimal volume of buffer A and subjected to an Ultrogel Aca 34 column (95×1.5 cm). Fractions of 3.5 ml were collected and tested for beauvericin synthesis.

Ion-exchange Chromatography on DEAE-trisacryl

Active fractions from the gel filtration step were pooled and applied to a DEAE-trisacryl column (8×1 cm) and washed with buffer A. Bound protein was eluted by an increasing gradient from 0~0.3 M KCl in buffer A. Active fractions were concentrated about 5-fold by ultrafiltration using Centricon ultrafiltration units (Amicon, Witten, FRG) and dialyzed against buffer A.

Sucrose Gradient Centrifugation

Further purification of the enzyme was done by sucrose gradient centrifugation (5~20%) in 50 mM Tris-HCl pH 7.2, 0.25 mM EDTA and 8 mM DTE. Centrifugation was carried out in a Beckman Ultracentrifuge at 4°C, 35,000 rpm for 20 hours.

Chromatographic Systems

For TLC Silica Gel 60 plastic sheets (Merck, Darmstadt, FRG) were used. Reaction products were analyzed using solvent system I: EtOAc - MeOH - H₂O (100:5:1); and solvent system II: BuOH - AcOH - H₂O (4:1:1).

ATP-pyrophosphate Exchange

This was carried out as described previously⁶⁾.

Liberation of Thioesterified Substrates

8~15 μg of enzyme were incubated in the presence of 0.5 μCi Phe or HYIV, 6 mM ATP, 12 mM Mg(OAc)₂ in a total volume of 115 μl. After incubation for 10 minutes 2 ml of 7% TCA was added and the precipitate was pelleted by centrifugation. Precipitates were washed 3 times with 7% TCA and twice with EtOH. After drying, pellets were dissolved in 60 μl of performic or formic acid and incubated at 60°C for 30 minutes. Samples were then evaporated to dryness under reduced pressure, dissolved in 30 μl of formic acid and applied to TLC plates (solvent system II).

Molecular Mass Determination

The molecular mass of the native enzyme was determined by gel filtration on an Ultrogel Aca 34

column (95 × 1.5 cm) which was calibrated with enniatin synthetase (250 kdaltons), gramicidin S synthetase II (280 kdaltons) and gramicidin S synthetase I (100 kdaltons) as standard proteins. The molecular mass of the denaturated enzyme was determined by SDS gel electrophoresis¹⁰. Standard proteins used were; aldolase (40 kdaltons), ovalbumin (43 kdaltons), catalase (60 kdaltons), bovine serum albumine (BSA)-monomer (68 kdaltons), phosphorylase b (93 kdaltons), β -galactosidase (130 kdaltons), BSA-dimer (136 kdaltons), myosin (200 kdaltons), enniatin synthetase (250 kdaltons) and gramicidin S synthetase II (280 kdaltons).

Protein Determinations

Protein concentrations were determined by the BRADFORD procedure using bovine serum albumin as a standard¹¹.

Pantothenate Assay

This assay was performed as described by PUGH and WAKIL using *Lactobacillus plantarum* ATCC 8014 as test organism¹².

Results

Preparation of Enzyme

Beauvericin synthetase from *B. bassiana* was purified to near homogeneity in 6 steps (Table 1) including poly(ethyleneimine) and $(\text{NH}_4)_2\text{SO}_4$ precipitation, gel filtration on Ultrogel AcA 34, anion exchange chromatography and sucrose gradient centrifugation. After the DEAE step, purity of the enzyme was already 95% as judged by gel scanning. Traces of contaminating protein could be removed by the sucrose gradient step (Fig. 2). The enzyme could be stored at -80°C without significant loss of activity in the presence of 20% glycerol, 2.5 mM EDTA and 5 mM DTE.

Molecular Mass Determination

The molecular mass of the native enzyme was determined by gel filtration. A value of about 250 kdaltons was obtained. The molecular mass of the denaturated enzyme was determined by SDS gel electrophoresis. As can be seen, from this method a similar molecular mass as from the gel filtration was obtained (Fig. 2). The mobility of beauvericin synthetase was not changed by treatment with excessive amounts (0.2 M) of reducing agents like mercaptoethanol or DTE or detergents like SDS at 100°C .

Influence of pH and Temperature

The optimum pH for beauvericin formation was found to be pH 7.2 in phosphate buffer. At pH 6.8 and 7.5 activity was about 80% of the optimum value. Optimum temperature was found to be $25\sim 27^\circ\text{C}$. Increase of temperature above 30°C lead to rapid loss of activity, which was irreversible.

Table 1. Purification of beauvericin synthetase.

Step	Volume (ml)	Total protein (mg)	Activity (pKat)	Specific activity (pKat/mg)	Yield (%)
Crude extract	179.0	2,264.3	146.8	0.065	100
Poly(ethyleneimine) precipitation	167.0	796.9	127.7	0.16	87
$(\text{NH}_4)_2\text{SO}_4$ precipitation	12.2	95.7	68.5	0.716	46.7
Gel filtration on Ultrogel AcA 34	76.0	56.8	54.2	0.954	37
DEAE-trisacryl	8.6	16.6	25.4	1.53	17.3
Sucrose gradient centrifugation	4.0	5.2	9.1	1.75	6.2

8.25 g of lyophilized mycelium were used.

Studies on Substrate Activation

In further experiments we examined the activation mechanisms of beauvericin synthetase. From a number of peptide synthetases it is known that the activation of substrates occurs as thioesters *via* adenylation. Similarly beauvericin synthetase is able to catalyze ATP/pyrophosphate exchange reactions dependent on the primary precursors of beauvericin, D-HYIV and L-Phe. Incubation of the enzyme with radio-labeled D-HYIV or L-Phe in the presence of ATP/Mg²⁺ and subsequent treatment with TCA yielded covalently labeled protein (not shown).

As can be seen in Fig. 3, the covalently bound substrates could be split off the enzyme by treatment with performic acid and separated by TLC. Lane A, Fig. 3a shows the control experiment, where formic acid was used, and lane B release of [¹⁴C]Phe by performic acid oxidation. When the enzyme was loaded with [¹⁴C]Phe in the presence of AdoMet almost exclusively NMe-Phe was released (lane C). In Fig. 3b, the liberation of [¹⁴C]HYIV is shown.

These findings indicate an activation of the substrate hydroxy- and L-amino acids *via* thioester linkages. Another hint for the existence of thioesterified intermediates during beauvericin synthesis comes from the behavior of the enzyme towards specific thiol group blocking agents such as iodoacetamide, *N*-ethylmaleimide and *p*-chloromercuribenzoate. These substances exhibited a strong inhibitory influence on enzyme activity by preventing the ability to form covalent enzyme-substrate complexes (not shown).

Presence of 4'-Phosphopantetheine in Beauvericin Synthetase

Purified enzyme from the sucrose gradient step was used to determine the 4'-phosphopantetheine content using *L. plantarum* as a test organism. After treatment of the protein with KOH and subsequent incubation with alkaline phosphatase, growth of *L. plantarum* was observed, indicating the presence of 4'-phosphopantetheine. Without alkaline phosphatase treatment no growth was obtained.

Substrate Specificity of Beauvericin Synthetase

Beauvericin belongs to the class of cyclodepsipeptides with enniatin-like structure. It contains the aromatic amino acid L-Phe instead of the aliphatic residues in the different enniatins. Therefore it was of interest to study the specificity of this enzyme towards analogue amino acids to elucidate

Fig. 2. SDS-Gel electrophoresis of protein samples from different purification steps of beauvericin synthetase.

Lane A shows gramicidin S synthetase II (280 kdaltons) and enniatin synthetase (250 kdaltons). Lanes B, C and D show samples of crude extract, DEAE ion exchange chromatography and sucrose gradient centrifugation, respectively. Bars represent positions of other marker proteins. Staining was done with coomassie blue.

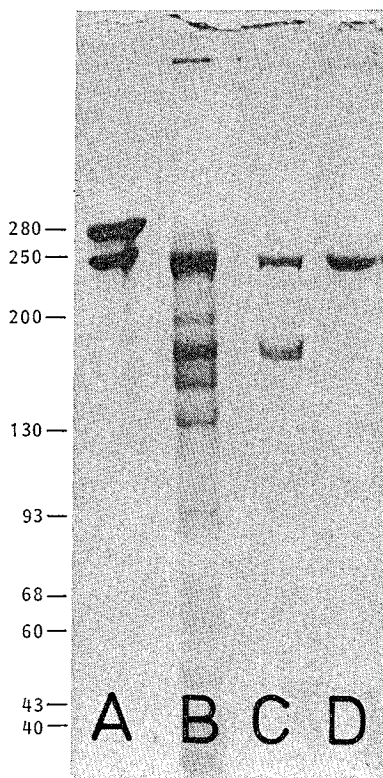


Fig. 3. Liberation of thioesterified Phe and HYIV from beauvericin synthetase by treatment with performic acid.

(a) Liberation of covalently bound [14 C]Phe and [14 C]NMePhe by treatment with performic acid after labeling of the enzyme with [14 C]Phe (lane C) or with [14 C]Phe in the additional presence of AdoMet (lane B). Lane A represents a control experiment with formic acid instead of performic acid using the [14 C]Phe labeled enzyme. TLC was carried out as described under Materials and Methods.

(b) The same experiment as described under 3a using [14 C]HYIV instead of [14 C]Phe. Lane A shows the control experiment using formic acid. Lane B shows liberation of [14 C]HYIV by performic acid treatment. Exposure of the chromatograms to X-ray film was for 7 days.

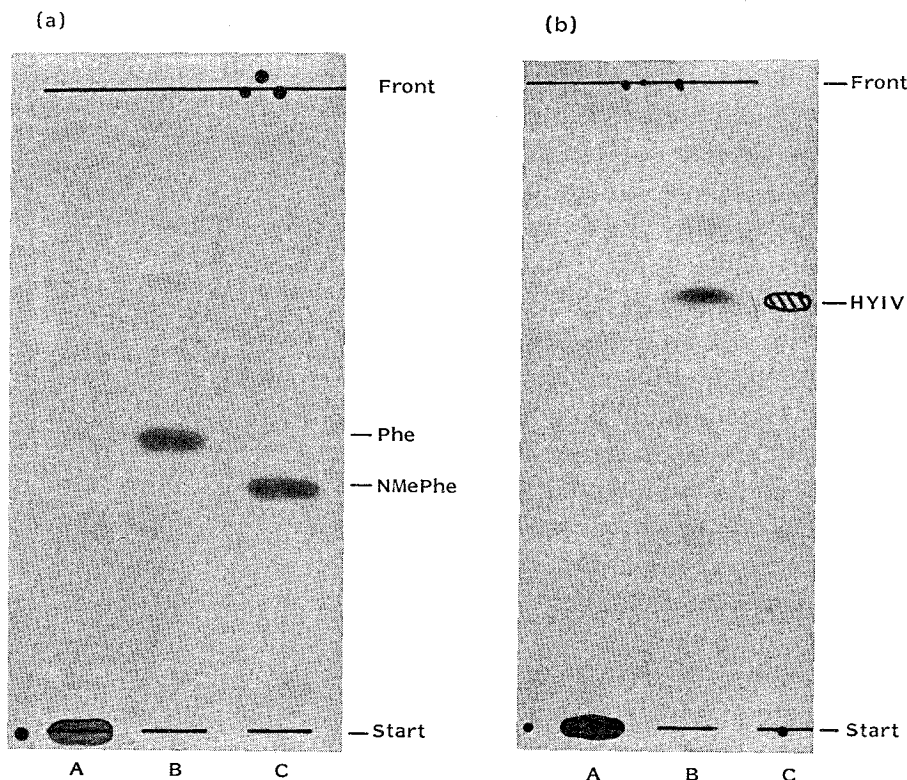


Table 2. Incorporation of different amino acids into new depsipeptides by beauvericin synthetase.

Amino acid	Relative incorporation rate (%)	Rf value ^a
L-Phenylalanine	100	0.40
D-Phenylalanine	0	—
L-Phe (minus AdoMet) ^b	15	0.18
<i>o</i> -Fluoro-DL-phenylalanine	57	0.60
<i>m</i> -Fluoro-DL-phenylalanine	30	0.51
<i>p</i> -Fluoro-DL-phenylalanine	28	0.38
Allylglycine	37	0.17
Leucine	17	0.18
Norleucine	15	0.20
Isoleucine	26	0.29
<i>o</i> -Tyrosine	2	0.33
β -Phenylserine	35	0.48
2-Amino-4-methylhex-4-enoic acid	22	0.36
Valine	0	0.68
Norvaline	14	0.66

^a Solvent system I.

^b Product formed was demethylbeauvericin (see Fig. 4).

—: No product was formed.

Table 3. K_m values of beauvericin synthetase and the related enniatin synthetase.

Substrate	Beauvericin synthetase K_m (μM)	Enniatin synthetase K_m (μM)
D-HYIV	1.0	5.0
AdoMet	3.5	7.0
L-Phe	10.0	^a
ATP	100.0	350
L-Val	^a	80

^a No product formation.

differences between beauvericin synthetase and enniatin synthetase. The purified enzyme was incubated in the presence of D-HYIV, ATP/Mg²⁺, [methyl-¹⁴C]AdoMet and the new amino acid to be incorporated. Products formed were extracted with EtOAc and subjected to TLC. The relative incorporation rates (compared to Phe) of different substrate amino acids are shown in Table 2. As can be seen, L-Phe easily could be replaced by other hydrophobic amino acids such as allylglycine, leucine, norleucine, isoleucine and 2-amino-4-methylhex-4-enoic acid. Also the *ortho*-, *meta*- and *para*-fluoro derivatives of DL-phenylalanine could be incorporated. Valine, the constituent amino acid of the enniatin B molecule was not accepted by the enzyme. However in the case of leucine and isoleucine the corresponding enniatins C and A were formed.

The N-Methylation Step

As shown previously in the case of the depsipeptide enniatin B in the absence of AdoMet during the biosynthetic process demethylnniatin is formed. The same holds true for the beauvericin synthetase system. Fig. 4 shows the formation of demethylbeauvericin when the enzyme was incubated with all necessary substrates except AdoMet. Identification of the unmethylated product was carried out as described³⁾.

Kinetic Measurements

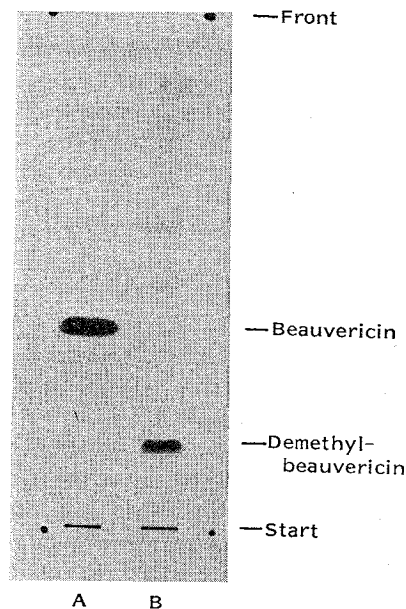
For determination of the K_m values under saturating conditions for the different substrates of beauvericin synthetase initial rate studies were done. The initial reaction rate was measured at varying concentrations of one substrate with fixed saturating concentrations of the others. K_m values were determined from double reciprocal plots and are listed in Table 3. For comparison the K_m values of enniatin synthetase, a similar multifunctional enzyme system from *Fusarium oxysporum*, are also given⁴⁾.

Discussion

Beauvericin synthetase, a multifunctional enzyme from *B. bassiana* was purified to near homogeneity. The enzyme strongly resembles the enniatin synthetase system from *F. oxysporum* in its

Fig. 4. Formation of demethylbeauvericin.

Lane B shows the formation of demethylbeauvericin from [¹⁴C]Phe, ATP/Mg²⁺, D-HYIV (omission of AdoMet). Lane A: beauvericin. Exposure of the chromatogram to X-ray film was for 4 days.



molecular size and the reaction mechanism. It consists of a single polypeptide chain with a molecular mass of about 250 kdaltons. Beauvericin is synthesized from its precursors HYIV and Phe by a thio-template mechanism including *N*-methylation of the thioesterified substrate amino acid residues. Free *N*-methylamino acids are not accepted by the enzyme.

So far a number of similar *N*-methylation reactions have been described in procaryotic and eucaryotic systems like cyclosporin¹³⁾, actinomycin¹⁴⁾, echinomycin (U. KELLER; personal communication) and the enniatins⁵⁾. Therefore, this pathway of *N*-methylation seems to be of general importance in the synthesis of peptides and depsipeptides in nature.

The major difference between beauvericin and enniatin synthetase may lie in the specificity of the enzymes to their substrate amino acids. Beauvericin synthetase preferably accepts amino acids derived from Phe which has the highest efficiency of incorporation. Also a number of aliphatic hydrophobic amino acids were accepted, leading to new beauvericin-like depsipeptides. The incorporation rates strongly decrease with the length of the hydrophobic side chains as depicted in Table 2. Isoleucine still yielded 26% of the Phe product formation, whereas valine, lacking a further methyl group, does not respond at all. Comparison of *K_m* values of both enzymes shows that the affinities to the substrates AdoMet, HYIV and ATP lie in the same range, whereas valine (*K_m* 80 μ M) and Phe (*K_m* 10 μ M) are quite different. The relatively high affinity of beauvericin synthetase to Phe may explain the absence of hybrid beauvericins derived from leucine and isoleucine in *B. bassiana*, whereas enniatin synthetase in *F. oxysporum* allows formation of different hybrids like enniatins A₁, B₁ beside the main product enniatin B.

Studies with monoclonal antibodies to enniatin synthetase have shown that antibodies directed against the HYIV binding site or the *N*-methyltransferase site cross-react with beauvericin synthetase, whereas antibodies directed against the valine binding site did not show any reaction¹⁵⁾.

This indicates that beauvericin and enniatin synthetase seem to be very similar in their catalytic domains concerning the HYIV activation and the *N*-methylation site, however strongly differ in their amino acid recognition site(s).

Acknowledgments

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