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PARTIAL PURIFICATION, SPECIFICITY AND MECHANISM OF ACTION OF THE NISIN-INACTIVATING ENZYME FROM *BACILLUS CEREUS*

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SUMMARY

1. A 12–20-fold purification of the nisin-inactivating enzyme from *Bacillus cereus* has been achieved in yields of 25–40%.

2. The enzyme inactivated nisins A, B, C and E but not nisin D, and it was unable to inactivate the antibiotics penicillin, erythromycin, neomycin, streptomycin and tylosin. Since the enzyme also inactivates subtilin[1], the evidence suggests that the enzyme is specific for a particular group which occurs in the peptide antibiotics nisin and subtilin.

3. The K_m values for nisins A, B, C and E were $33 \cdot 10^{-3}$, $35 \cdot 10^{-3}$, $1.74 \cdot 10^{-3}$ and $1.26 \cdot 10^{-3}$ M, respectively. These results suggest stereochemical differences between the nisins.

4. Chemical studies of enzymically inactivated nisin showed that the C-terminal sequence of dehydroalanyllysine was reduced to alanyllysine and it is suggested, therefore, that the enzyme is a dehydropeptide reductase.

INTRODUCTION

Inactivation of the polypeptide antibiotic nisin by non-proteolytic enzymes from several species of Bacillus has been reported previously[1]. The enzymes from *Bacillus cereus* and *Bacillus polymyxa* inactivated the antibiotics nisin and subtilin but not other peptide antibiotics tested[1] and differed from the cell-wall lytic enzyme of STRANGE AND DARK[2] and from the spore-germinating enzyme of GOULD AND HITCHINS[3]. In a preliminary study of the mechanism of enzymic inactivation of nisin[4], no major molecular differences were observed in enzyme-inactivated nisin compared with nisin from incubated controls. GROSS AND MORRELL[5] showed that nisin could be inactivated chemically by the hydrolytic cleavage of the C-terminal sequence of dehydroalanyllysine and recently GROSS *et al.*[6] showed that the same C-terminal sequence occurs in the antibiotic subtilin. The present paper reports the partial purification and substrate specificity of the nisin-inactivating enzyme from *B. cereus* and provides evidence in favour of the hypothesis that the enzyme acts as a dehydropeptide reductase, of which a preliminary account has been published elsewhere[7].

MATERIALS AND METHODS

Cultivation of the organisms

Stock cultures of *B. cereus* (NCDO 1937) were maintained on slopes of Tryptone Soya agar at 4°. Organisms were grown in 60 l fermenters at 30° in Tryptone Soya broth, with forced aeration at 30 l/min for the first 8 h and then 60 l/min (agitator speed 250 rev./min). Foam was controlled with the use of Silicone RD antifoam (Midland Silicones Ltd., Barry, Glamorgan, United Kingdom). The fermentation was allowed to proceed until sporulation was advanced and liberation of the endospores was commencing (about 42 h)[1]. The broth cultures were cooled rapidly to 2°, harvested by centrifugation in a Sharples centrifuge and washed once with buffer (0.1 M citric acid + 0.2 M Na_2HPO_4 (pH 7.0)). After washing, the cell paste was freeze-dried; the yield of dried cells varied from 3.4 to 4.2 g/l medium.

Extraction of the enzyme

Freeze-dried cells (50 g) were ground for 5 min with an equal weight of levigated alumina (Norton Abrasives Ltd., Welwyn Garden City, United Kingdom) followed by extraction for 1 h at 37° with 1 l of buffer (0.05 M citric acid + 0.1 M Na_2HPO_4 (pH 6.5)). After cooling and centrifugation at 10 000 × *g* the supernatant solution was adjusted to 75% saturation with $(NH_4)_2SO_4$ and allowed to stand for 1 h at room temperature with 10 g Hyflo Super-cel 535. The precipitated proteins were separated by filtration and the filter cake was washed with 75% saturated $(NH_4)_2SO_4$ solution. The proteins were extracted with 0.05 M $NaHCO_3$ (4 × 50 ml) and the combined extracts were concentrated by overnight dialysis against an equal volume of 30% (w/v) Carbowax 4000. The concentrate was fractionated by precipitation with 2.5 vol. of ethanol at −20° and the protein precipitate was collected by centrifugation at 2500 × *g*. The precipitated proteins were extracted into a minimal volume of citrate–phosphate buffer (pH 6.8) and sterilized by membrane filtration. Aliquots of the sterilized solution were stored in sealed sterile ampoules at −20°. A summary of the partial purification achieved in a typical experiment is given in Table I.

Enzyme assay

The enzyme assay procedure and the units of activity have been described

TABLE I

PARTIAL PURIFICATION OF THE NISIN-INACTIVATING ENZYME

50 g freeze-dried cells were ground with alumina and extracted for 1 h at 37° with buffer (0.05 M citric acid + 0.1 M Na_2HPO_4 (pH 6.5)). Cell debris was removed by centrifugation at 10 000 × *g* to yield the crude cell-free extract, which was fractionated with $(NH_4)_2SO_4$ and ethanol.

Fraction	*Vol. (ml)*	*Total protein (mg)*	*Total activity (units)*	*Specific activity (units/mg of protein)*	*Yield (%)*	*Relative purity*
10 000 × *g* supernatant	750	2850	4050	1.4	100	1
75% $(NH_4)_2SO_4$-insoluble fraction	105	800	3045	3.8	75	3
2.5 vol. ethanol-insoluble fraction	52	55	1300	23.8	32	17

previously[1]. Incubation mixtures (9 ml) contained 1 μmole each of Ca^{2+} and Co^{2+}, 5000 units of commercial nisin (nisaplin) and a suitable aliquot of enzyme preparation (for the controls, an equal aliquot of heat-inactivated enzyme was used) in buffer (0.05 M citric acid + 0.1 M Na_2HPO_4 (pH 6.8)). The mixtures were incubated for 6 h at 30° and the reaction was stopped by the addition of 1 ml of 2 M HCl. The tubes were heated for 5 min at 100°, cooled and diluted for bioassay by the method of TRAMER AND FOWLER[8].

Michaelis constants

For the determination of Michaelis constants, purified preparations of nisins A, B, C, D and E (see below) were used as substrates. Calculations of the Michaelis constants assume a molecular weight of 7000 for the nisin dimer[9], the rates of inactivation being derived from the observed losses in biological activities.

Chemical studies on inactivated nisin

Reaction mixtures containing 25 mg 'pure' nisin were incubated in 9 ml phosphate buffer (0.1 M, pH 6.8) containing 1 μmole each of Ca^{2+} and Co^{2+} and 0.5 mg enzyme protein for 72 h at 30°. The mixtures were acidified to pH 1, heated for 5 min at 100° and filtered through a 2-μ membrane filter (Millipore Ltd., London) to remove precipitated protein. Aliquots were taken for bioassay and nisin was recovered from the remaining acidified reaction mixtures by precipitation with cold (0°) trichloroacetic acid (final concentration 10% (w/v)). After washing twice with cold 10% (w/v) trichloroacetic acid the nisin precipitates were dissolved in 1 M HCl or in 1 M HCl in glacial acetic acid and stored at 4°.

Partial hydrolysis of the nisin preparations was done by heating nisin dissolved in HCl–acetic acid for 10 min at 110° (ref. 5). Heated and unheated preparations were freeze-dried and dissolved in 0.2 M acetic acid for chromatographic examination. The carboxymethylcysteine derivative of nisin was prepared essentially as described by GROSS AND MORRELL[10] except that the addition product was separated from excess methylmercaptoacetate by precipitation of the nisin with trichloroacetic acid (see above). High-voltage electrophoresis of hydrolysed samples of this material were carried out on paper using a formic acid–acetic acid buffer (31.2 ml formic acid + 59.2 ml acetic acid diluted to 1 l with water, pH 1.9) with a potential of 60 V/cm for 35 min. After drying, the electrophoretograms were developed using ninhydrin–collidine reagent[11]. The electrophoretograms were quantified by densitometry using a Chromoscan (Joyce Loebl Ltd., Gateshead, England). A standard curve was prepared by electrophoresis of aliquots of a standard solution of *S*-carboxymethylcysteine mixed with a hydrolysate of normal nisin. The recovery of *S*-carboxymethylcysteine was determined after hydrolysis of a mixture of *S*-carboxymethylcysteine and nisin.

The addition product of nisin and [^{14}C]cysteine was prepared by incubation of 1.5 nmoles nisin for 24 h at 30° with 2.5 μC of [^{14}C]cysteine in phosphate buffer (0.1 M, pH 7.0). 100 μg carrier nisin were added and the nisin was precipitated with trichloroacetic acid (final concentration 10% (w/v)). The precipitate was washed 3 times with cold 10% (w/v) trichloroacetic acid, and then sequentially with 2% (w/v) Na_2SO_4, acetone and ether. The dried residues were dissolved in 1 M HCl in glacial acetic acid and aliquots were counted using the cocktail according to BRAY[12] in a Corumatic scintillation counter (Tracerlab Ltd., Weybridge, United Kingdom), a

correction being applied for quenching. Carrier nisin (5 mg) was added to the remaining labelled material and the mixture was partially hydrolysed in 1 M HCl in glacial acetic acid (see above) and examined by gel filtration.

Gel-filtration chromatography

Nisin preparations were chromatographed on columns of Sephadex G-25 (fine grade, 80 cm × 2.5 cm) using 0.2 M acetic acid as eluent. The absorbance of fractions (3 ml) was determined at 250 nm and radioactivity was determined by scintillation counting, the counts being corrected for quenching.

Chemicals

Antibiotics and enzyme substrates were obtained from the following sources: Benzyl penicillin and streptomycin (Glaxo Laboratories Ltd., Greenford, England); chloramphenicol and neomycin (Boots Pure Drug Co. Ltd., Nottingham, England); erythromcyin (Abbott Laboratories Ltd., Queenborough, England); chlortetracycline (Lederle Laboratories, London); tylosin lactate was a gift from Eli Lilly and Co., Indianapolis 6, U.S.A.; nisaplin (1 unit nisin/μg) and purified nisin (30 units/μg) were gifts from Aplin and Barrett Ltd., Yeovil, England. Nisin A (23.8 units/nmole), nisin B (25.3 units/nmole), nisin C (9.9 units/nmole), nisin D (8.5 units/nmole) and nisin E (2.9 units/nmole) were isolated from purified nisin by counter-current distribution[9,13,14].

S-Carboxymethyl-L-cysteine was from British Drug Houses Ltd., Poole, England, and methylmercaptoacetate was supplied by Sigma Chemicals, London. Other chemicals were of analytical grade.

Radioactive amino acids

Uniformly labelled [^{14}C]cysteine (18.3 μC/μmole) was purchased from the Radiochemical Centre, Amersham, England.

RESULTS

Partial purification of the enzyme

The scheme of partial purification (see MATERIALS AND METHODS) permitted a 17-fold increase in the specific activity of the enzyme (Table I). In 15 separate purifications the increase in specific activity ranged from 12- to 20-fold in yields of 25–40%. Much of the ethanol precipitated protein appeared to be denatured since it was completely soluble only in alkaline solutions (pH > 10). Attempts to further purify the enzyme by gel filtration through columns of Sephadex G-100 and G-200 in various buffers were unsuccessful, the enzymic activity appearing in several overlapping peaks, thus suggesting polymerization of the enzyme or the presence of several different enzymes with similar biological activities. This aspect is being investigated further.

Storage experiments showed that although freeze-dried or acetone-dried cell pastes[1] retained their activity over a 3-year period at −20°, rapid and total inactivation of the partially purified enzyme preparation occurred on freeze-drying. Attempts to prolong the storage life by the use of adjuncts such as albumin and sucrose were unsuccessful. Membrane-filtered sterile solutions of the enzyme retained their activity over a period of 2 years when frozen rapidly and stored at −20°. The presence of Ca^{2+}

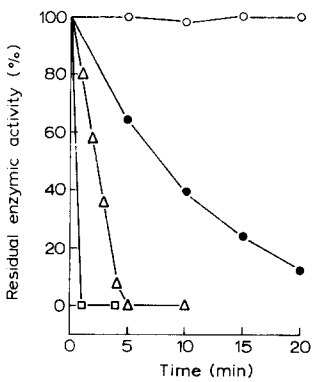

Fig. 1. Thermal stability of the enzyme. Preparations of the enzyme were held at 50° (○—○), 52° (●—●), 55° (△—△) and 60° (□—□). Samples were removed after various time periods, cooled rapidly to 2° and then assayed for residual enzymic activity.

and either Co^{2+} or Mg^{2+}, which are required for maximal activity[1], did not influence the stability of the enzyme during storage. The enzyme was stable for 1 h at 50°, but was totally inactivated after 1 min at 60° and 5 min at 55° (Fig. 1).

Substrate specificity of the enzyme

The enzyme did not inactivate benzyl penicillin, chlortetracycline, erythromycin, neomycin, streptomycin nor tylosin. Earlier observations[1] had shown that the enzyme was unable to inactivate bacitracin, gramicidin S and polymyxin A. Although nisin is slowly inactivated by subtilopeptidase A (EC 3.4.4.16)[1] and α-chymotrypsin (EC 3.4.4.5)[15], early work on the enzyme specificity had shown that the enzyme was non-proteolytic[1] and evidence was obtained to suggest that the inactivated nisin molecule retained its basic molecular size[4].

The enzyme inactivated nisins A, B, C and E but failed to inactivate nisin D,

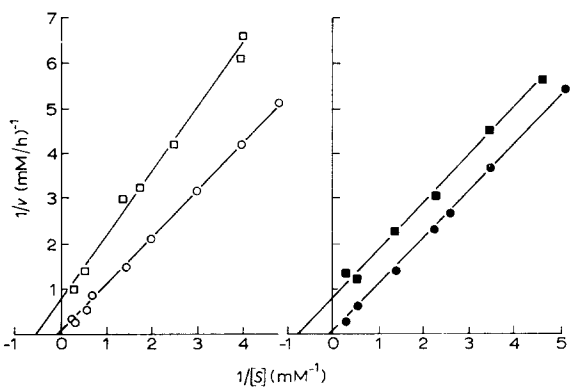

Fig. 2. Determination of Michaelis constants. The initial rate of inactivation (μmoles/ml per h) at various substrate concentrations (μmoles/ml) was determined at pH 7.0 and 30° for nisin A (○—○), nisin B (●—●), nisin C (□—□) and nisin E (■—■) from the observed loss of antibiotic activity. The best straight lines were derived by the method of least squares.

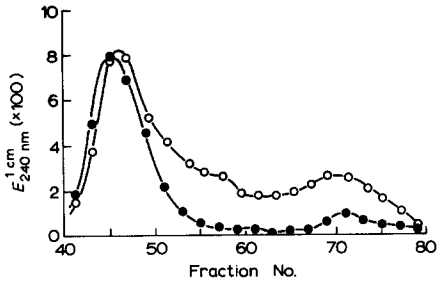

Fig. 3. Gel filtration of enzyme-inactivated nisin. Inactivated nisin in 0.2 M acetic acid was examined by chromatography on a column of Sephadex G-25 (80 cm × 2.5 cm) using 0.2 M acetic acid as eluent. The curves illustrate the patterns for nisin before (●—●) and after (○—○) partial hydrolysis (1 M HCl in glacial acetic acid, 10 min at 110°). Similar curves were obtained for non-inactivated, incubated nisin controls.

the amino acid composition of which differs from those of the other nisins[14]. At substrate concentrations ranging from 0.25 to 5.0 mM (as the dimer) the mean relative rates of biological inactivation of nisins A, B, C, D and E were 100, 102, 50, 0 and 66, respectively. Michaelis constants, determined by the method of DIXON[16] (Fig. 2), were $1.74 \cdot 10^{-3}$ and $1.26 \cdot 10^{-3}$ M for nisins C and E, respectively. Approximate K_m values of $33 \cdot 10^{-3}$ and $35 \cdot 10^{-3}$ M for nisins A and B, respectively, were obtained but these results cannot be considered accurate since at these concentrations nisin is insoluble at the pH of the reaction mixture. The K_m value for the enzyme against subtilin could not be determined due to lack of material.

Mechanism of the enzymic inactivation

Earlier investigations had shown no change in the sedimentation coefficient,

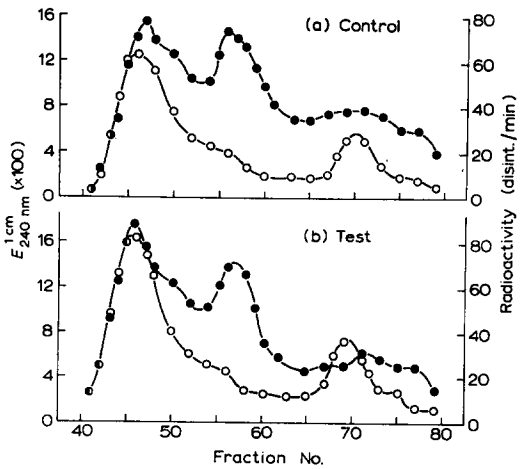

Fig. 4. Gel filtration of the [^{14}C]cysteine-labelled nisin addition product after partial hydrolysis. The ^{14}C-labelled nisin was partially hydrolysed (1 M HCl in glacial acetic acid; 110° for 10 min) with about 5 mg carrier nisin and then passed through a column of Sephadex G-25 (80 cm × 2.5 cm) using 0.2 M acetic acid as eluent. Curve a, addition product of incubated control nisin + cysteine; Curve b, enzyme-inactivated nisin + cysteine; ○—○, $E^{1\,cm}_{240\,nm}$; ●—●, disint./min corrected for background.

chromatographic or electrophoretic mobility, C- and N-terminal amino acids of enzyme-inactivated nisin compared with incubated controls[4]. The report of a common C-terminal sequence of dehydroalanyllysine in both nisin and subtilin[6] (the only two antibiotics which are inactivated by the enzyme) suggested that the enzyme might act as a dehydropeptide reductase or as a dehydropeptidase.

Bioassay of incubated pure nisin preparations showed a mean inactivation of 76%. Chromatographic examination of inactivated nisin and nisin controls on Sephadex G-25 provided no evidence for the release of the C-terminal dehydroalanyllysine sequence as pyruvyllysine (Fig. 3) and precluded the possibility that the enzyme acted as a dehydropeptide hydrolase. No differences were observed between the nisin preparations after partial hydrolysis (Fig. 3) although when the 'pyruvyllysine' peak was concentrated by freeze-drying and examined by thin-layer chromatography on Silica gel G (butan-1-ol–acetic acid–water (80:20:20, by vol.) a mixture of pyruvyllysine and another peptide was observed. Chromatography of hydrolysates of the material recovered from the pyruvyllysine peaks showed the presence of a mixture of alanine and lysine from inactivated nisin but only lysine in the material from the control nisin.

Quantitative high-voltage electrophoresis of a hydrolysate of the nisin preparations which had been treated with methylmercaptoacetate showed a decrease in the amount of *S*-carboxymethyl-L-cysteine in the inactived nisin (Table II). Recovery of

TABLE II

SUMMARY OF EXPERIMENTAL RESULTS OBTAINED WITH ENZYME-INACTIVATED AND INCUBATED CONTROL PREPARATIONS OF NISIN

For experimental details see text.

Preparations	*Biological activity*		*[^{14}C]Cysteine-binding*		*S-Carboxymethyl-cysteine*	
	units/ml $\times 10^{-3}$	%	*Disint./min*	%	*moles/mole nisin*	%
Control nisin ($n = 6$)	350	100	18 040 (17 200–18 400)	100	1.70 (1.65–1.73)	100
Inactivated nisin ($n = 6$)	82.5	24	13 950 (12 900–14 600)	77.4	1.08 (1.04–1.11)	63.5
Theoretical	—	24	—	73.6	—	62

S-carboxymethyl-L-cysteine was 91%. The uncorrected concentration of carboxymethyl-L-cysteine in the nisin hydrolysates was 1.7 residues/mole for the control material which is identical to that reported by GROSS AND MORRELL[10]. No differences were observed in the level of β-methyl-*S*-carboxymethylcysteine (0.9 residue/mole nisin).

Binding of [^{14}C]cysteine to the α,β-unsaturated amino acids of nisin occurred linearly over a period of 24 h. When inactivated nisin was incubated with labelled cysteine the mean binding was 13 950 disint./min whereas the control nisin bound 18 040 disint./min (Table II). Assuming that only one of the three α,β-unsaturated linkages was reduced by the enzyme and that the nisin was 76% inactivated, the expected binding of cysteine would be (24 × 3)/100 + (76 × 2)/100 *i.e.* 74.6%. The

inactivated nisin was observed to bind 77.4% of the amount of cysteine bound by the control nisin. When the ^{14}C-labelled nisin was subjected to partial hydrolysis in the presence of carrier nisin, the gel-filtration eluate pattern showed a reduction in the ^{14}C content of the 'pyruvyllysine' peak in the test relative to the control (Fig. 4).

DISCUSSION

The evidence presented on the mechanism of enzymic inactivation of nisin favours the hypothesis that the enzyme is a dehydroalanine reductase. It is hardly surprising, therefore, that it is specific for nisin and subtilin, both of which have a C-terminal sequence of dehydroalanyllysine. The presence of dehydropeptides in other proteins has recently been reported[18] and it will be interesting to determine whether the enzyme is able to reduce these unsaturated residues. It was suggested previously[1] that the enzyme probably has some function other than that of the inactivation of peptide antibiotics and, as suggested by GROSS *et al.*[6], it is quite possible that many other microbial and even animal peptides may contain α,β-unsaturated amino acid residues. Since nisin D is not inactivated it is possible that this peptide does not contain the terminal dehydroalanyllysine sequence. Investigations to confirm this hypothesis are currently proceeding.

Investigations of the molecular weight distribution of nisin showed that it normally occurs as a dimer[9]. Recent investigations of the amino acid composition of several different nisins suggest that the dimeric form may consist of two dissimilar peptide chains[17]; the differences between the peptide chains would be sufficient to affect the stereochemical configuration of the dimer and might be expected to influence both the biological activity of the nisins and also the affinity of the enzyme for the different nisins. The observed K_m values are consistent with this hypothesis. Since nisin is readily inactivated by partial hydrolysis under relatively mild conditions, it is suggested that the C-terminal sequence is not tightly bound into the steric configuration of the nisin and is therefore available for enzymic reduction. The other α,β-unsaturated residues are doubtless masked within the molecule but are able to complex low-molecular-weight thiol derivatives such as cysteine and methylmercaptoacetate.

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