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PEPTIDASE ACTIVITY IN THE INNER MEMBRANE OF *PSEUDOMONAS AERUGINOSA*

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Summary

The location of peptidase activity within the cell envelope structure of *Pseudomonas aeruginosa* has been studied. Inner and outer membrane fractions were separated on the basis of buoyant density using two consecutive sucrose step gradients and identified on the basis of known components. The inner membrane was shown to contain peptidase activity while the outer membrane contained none. These data support the hypothesis that *P. aeruginosa* transports intact peptides.

In recent years it has been possible to utilize the peptide transport system to introduce deleterious and otherwise impermeable molecules into bacterial and fungal cells. The process has been termed 'illicit transport' [1] and has been demonstrated in *Escherichia coli*, *Salmonella typhimurium* and *Candida albicans* [1—3]. The need for this new approach towards the construction of antimicrobial agents has arisen with the development of strains of bacteria which are concomitantly resistant to several antibiotics.

Pseudomonas aeruginosa, a plasmid-bearing organism, is notorious for its ability to acquire resistance factors. The use of illicit transport may aid in the control of *P. aeruginosa* infections. As a first step toward this end, studies on the utilization of peptides by *P. aeruginosa* have been carried out by Miller and Becker [4]. They found that peptidase activity on the model substrate tri-

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methionine was associated with the membrane fraction of cell lysates. In the present study, we present evidence that this peptidase is a component of the inner membrane.

P. aeruginosa strain RM46 was used throughout these studies [4,5]. Stock cultures were maintained as dilute suspensions in Luria Complete Broth as described elsewhere [4]. Membrane preparations were prepared from cultures grown overnight on *Pseudomonas* minimal medium [4] or Luria Complete Broth at 37°C with aeration. Filter-sterilized glucose and methionine were added to the *Pseudomonas* minimal medium at concentrations of 0.4% and 50 μg/ml, respectively. Cultures used for the separation of inner and outer membranes were grown on Luria Complete Broth and those used for crude preparation of total membranes were grown on *Pseudomonas* minimal medium.

Total membrane preparations, washed by resuspension in saline, were obtained by the method described by Miller and Becker [4]. These preparations were assayed for their ability to liberate amino acids from various tripeptides. Assays were carried out by the chromatographic methods described elsewhere [4]. The preparations effectively degraded trimethionine, trimethionine methyl ester, acetyltrimethionine, trilysine, trileucine and triglutamic acid. 100% of the amino acids were liberated from 0.1 mg of tripeptide after incubation for 1.5 h at 37°C with 0.1 ml of a membrane preparation containing approx. 1 mg of protein.

Separation of inner and outer membranes was accomplished by the method of Hancock and Nikaido [6]. Cells were lysed using the French pressure cell and cell debris was removed by centrifugation. 5—6 ml of the lysate were then layered onto a sucrose step gradient that contained 1 ml 70% (w/v) sucrose and 6 ml 15% (w/v) sucrose, and the tube was centrifuged at 39 000 rev./min in a Beckman type SW41 rotor for 1 h. After centrifugation, membrane was found at the interface between the 15 and 70% sucrose. Peptidase activity was determined by the coupled enzyme assay described by Miller and Becker [4] after the gradient was fractionated into 22 parts. Approx. 70% of the total observed activity was located at the interface between the two concentrations of sucrose and those fractions were pooled and placed in a second sucrose step gradient.

The second sucrose gradient consisted of 1 ml 70% (w/v) sucrose, 3 ml 64% (w/v) sucrose, 3 ml 58% (w/v) sucrose and 3 ml 52% (w/v) sucrose. This gradient was centrifuged overnight (16 h) at 39 000 rev./min in a Beckman type SW 41 rotor. Fractions (0.6 ml) were collected from the top of the gradient. The second sucrose gradient separated the membrane fraction into four bands, as previously reported [6]. The outer membrane (band IV) was found within the high density portion of the gradient while the inner membrane (band I) was in the lower density region. The vast majority of peptidase activity was found to be associated with band I (Fig. 1A). The identity of band I as the inner membrane was confirmed by demonstrating the presence of succinate dehydrogenase, a component of the inner membrane of *E. coli* and *P. aeruginosa* (Fig. 1B). Band IV was identified as containing outer membrane components by demonstrating the presence of bacteriophage E79 receptor proteins, an outer membrane component of *P. aeruginosa* [7], and the absence of succinate dehydrogenase (Fig. 1B).

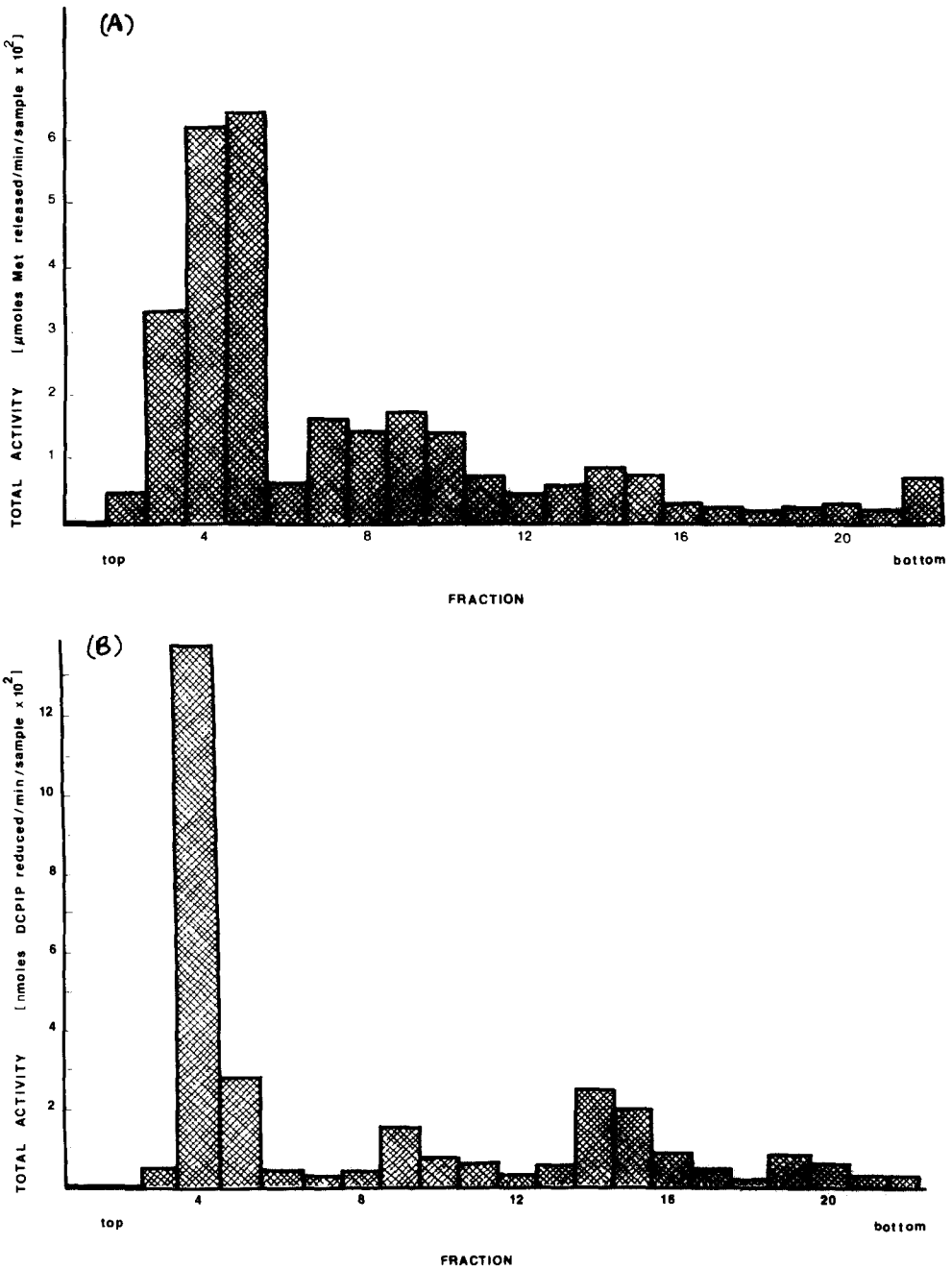

Fig. 1. Profiles of peptidase and succinate dehydrogenase activity in the second sucrose step gradient. After centrifugation, the gradient was divided into 22 fractions each of 0.6 ml by collection from the top of the tube (see text for additional details). Four visible bands of membrane material were obtained and labeled band I (fractions 3—5), band II (fractions 9—10), band III (fractions 13—15) and band IV (fractions 19—21). (A) Peptidase activity on trimethionine in the second sucrose step gradient. Each fraction was assayed for peptidase activity as described by Miller and Becker [4]. (B) Succinate dehydrogenase activity in the second sucrose step gradient. The reduction of dichlorophenol indophenol by this enzyme was determined spectrophotometrically at a wavelength of 600 nm as described by Hancock and Nakaido [6].

TABLE I

RELATIVE ACTIVITY OF PEPTIDASE AT VARIOUS STAGES OF ISOLATION

Protein determination was by the method of Lowry et al. [9] or that of Bradford [10]. Bovine serum albumin was used as a standard. The two methods gave similar results.

Fraction	Protein (mg/ml)	Volume (ml)	Spec. act. of peptidase (Units/mg protein) *
Cellular lysate	53.3	6	15
First gradient (pool) **	26.8	3	15
Second gradient (band I) ***	5.5	0.6	100

* A unit is defined as the amount of protein needed to liberate 1 μmol methionine from $(Met)_3$ in 1 min at 37°C.

** This pool was the bottom 3 ml of the gradient which contained the membrane fraction; see text.

*** Band I contained inner membrane; see Fig. 1A.

Between bands I and IV, two additional bands appeared that have been shown to contain a mixture of inner and outer membrane components by Hancock and Nikaido [6]. Each of the four bands was assayed for 2-keto-3-deoxyoctonate, a component of the outer membrane [8]. The majority of the 2-keto-3-deoxyoctonate was in band III, although none of this outer membrane component was associated with band I. This differs somewhat from the reports of Hancock and Nikaido [6] who found the largest amount of 2-keto-3-deoxyoctonate in band IV.

The peptidase found in isolated inner membrane (band I) was purified approx. 7-fold (Table I). When trimethionine was used as a substrate, the apparent K_m of the peptidase activity in the inner membrane was $9.4 \cdot 10^{-4}$ M and the apparent V was 2.7 mol/min. The optimum pH for peptidase activity associated with the inner membrane was between 7.5 and 8.0, while peptidase activity from the unfractionated cell envelope had an optimum at pH 7.5. Peptidase activity from band I was stimulated by the addition of Mg^{2+} and Mn^{2+} to the reaction mixture (optimum final concentration was approx. 17 mM). Addition of 0.1 mM Fe^{2+} to the reaction mixture inhibited 90% of the observed peptidase activity.

p-Phenylmethylsulphonyl fluoride, a protease inhibitor, was added to the lysate during the inner and outer membrane separation procedure. It was therefore prudent to determine that peptidase activity was not affected by *p*-phenylmethylsulphonyl fluoride. Total membrane was isolated as described above and treated with *p*-phenylmethylsulphonyl fluoride at a concentration comparable to that used in the inner and outer membrane separation procedure. The peptidase activity of this preparation was compared to the activity of an untreated sample. 96% of the trimethionine peptidase activity remained after *p*-phenylmethylsulphonyl fluoride treatment.

The trimethionine peptidase activity of the cell envelope structure of *P. aeruginosa* strain RM46 has been found to be associated with the inner membrane. Whether the peptidase activity associated with the inner membrane represents one or a number of enzyme species is not yet resolved. It seems likely that the active site of the inner membrane-bound peptidase(s) faces the cytosol. This conclusion is based on the fact that *P. aeruginosa* strain RM46 will not grow on

the modified peptide acetyltrimethionine [$Ac(Met)_3$] [4] although this peptide is broken into its constituent amino acids by incubation with membrane fractions. Based on the fact that larger peptides such as $(Met)_5$ (mol. wt. 674) are utilized by this strain as a source of methionine [4], we can safely assume, although it has not been specifically demonstrated, that $Ac(Met)_3$ (mol. wt. 453) has free access to the inner membrane. If the active site of the enzyme were facing the periplasmic space, $Ac(Met)_3$ would be broken down into methionine residues and transported as such. In this event, the methionine requirement of this strain would be met, and it would grow.

The data presented in this paper indicate that trimethionine is transported as an intact peptide. Extracellular breakdown of the peptide with subsequent transport of amino acids has been ruled out. Peptide transport through the cellular membrane could proceed by one of the two mechanisms. Either the peptide may be broken down by a peptidase located in the inner membrane after transport of the intact molecule, or it may be cleaved simultaneously with transport.

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