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ENZYMATIC HYDROLYSIS OF ENTEROCHELIN AND ITS IRON COMPLEX IN *ESCHERICHIA COLI* K-12

PROPERTIES OF ENTEROCHELIN ESTERASE

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Summary

Properties of the enzyme which hydrolyses enterochelin (a cyclic trimer of 2,3-dihydroxy-*N*-benzoyl-L-serine) to 2,3-dihydroxybenzoylserine have been investigated with a view to resolving discrepancies between earlier reports. Enterochelin esterase, previously reported to consists of two components (O'Brien, I.G., Cox, G.B. and Gibson, F. (1971) Biochim. Biophys. Acta 237, 537—549), has been shown to be fully active in the absence of the so-called A component. The hydrolase described previously (Bryce, G.F. and Brot, N. (1972) Biochemistry 11, 1708—1715) as being able to break down enterochelin but not its iron complex, ferric-enterochelin, appears to be identical with the B component of enterochelin esterase.

The single component enterochelin esterase corresponding to what was previously described as component B, hydrolyses both enterochelin and ferric-enterochelin. Under the assay conditions used, enterochelin is hydrolysed 2.5 times faster than the complex. Enzymatic activity is inhibited by *N*-ethylmaleimide and is lost rapidly at 37°C. Activity is stabilized in the presence of ferric-enterochelin, enterochelin, dithiothreitol or certain protein fractions.

Introduction

Enterochelin, a cyclic trimer of 2,3-dihydroxy-*N*-benzoyl-L-serine, is a component of a system for transporting iron in *Escherichia coli* K-12 [1]. It is also synthesised by *Klebsiella pneumoniae* (formerly *Aerobacter aerogenes*) [2] and by *Salmonella typhimurium* [3], and has also been called enterobactin [3]. Under conditions of iron deficiency, *E. coli* cells synthesize enterochelin and excrete it into the growth medium. Here a stable iron · enterochelin complex (ferric-enterochelin) is formed which is believed to be transported into the bacterial cytoplasm. Iron is released for cellular metabolism following enzy-

matic hydrolysis of the ligand to 2,3-dihydroxybenzoylserine. Synthesis of the esterase enzyme responsible for this hydrolysis is repressed in parallel with that of enterochelin synthetase when relatively low levels of iron are included in growth media [4].

Previous reports on the nature and specificity of this esterase are confusing. On one hand it has been reported that enterochelin esterase dissociates reversibly into two inactive components, designated A and B, during gel chromatography [4]. Cell extracts prepared from three mutant (*fes*B) strains unable to hydrolyse ferric-enterochelin have been shown to lack detectable levels of component B [5]. Enterochelin esterase present in *E. coli* cell extracts has been reported to hydrolyse ferric-enterochelin more rapidly than enterochelin [5]. In another report [6], a single component hydrolase has been described as being able to hydrolyse enterochelin but not ferric-enterochelin. In the present paper, the term enterochelin hydrolase is retained to identify the latter activity, although evidence is presented that it and component B of enterochelin esterase are in fact identical. This single component enzyme, for which continued use of the name enterochelin esterase is suggested, is able to hydrolyse both enterochelin and ferric-enterochelin. The reason for the descrepancies between our results and those reported previously seems to lie in the different assays used. A brief account of some of this work has been presented elsewhere [7].

Materials and Methods

Chemicals. Whenever possible, chemicals of analytical reagent quality were used. Ethyl acetate was redistilled before use. 2,3-Dihydroxybenzoic acid was obtained from the Aldrich Chemical Co., and L-[U-^{14}C]serine from the Radiochemical Centre, Amersham.

Preparation of enterochelin and 2,3-dihydroxybenzoylserine. Ferric-enterochelin was prepared by a method similar to that described by O'Brien et al. [4], and stored at −10°C. Supernatant liquid (10 l), obtained following centrifugation of stationary phase cultures of AN102, was cooled to 4°C and passed through a column (20 $cm^2 \times 15$ cm) of DEAE-cellulose equilibrated with 10 mM phosphate buffer (pH 7) and the absorbed ferric-enterochelin eluted with the same buffer containing 2 M NH_4Cl. Fractions containing the red ferric-enterochelin were pooled and washed twice with 0.1 vol. ethyl acetate, which was then discarded. The pH of the ferric-enterochelin solution was adjusted to approx. pH 1 with concentrated H_2SO_4 and the resulting mixture extracted three times with 0.1 vol. ethyl acetate. The ethyl acetate extracts were combined and washed three times with 1 vol. 0.1 M phosphate buffer (pH 7) to remove hydrolysis products of enterochelin. The concentration of enterochelin in the organic phase was determined spectrophotometrically using $E_{mM}^{316\,nm} = 9.39$ [2] and a solution of 1 mM ferric-enterochelin prepared by evaporating the ethyl acetate over the appropriate volume of 0.1 M phosphate buffer (pH 7) containing 1.05 mM $FeSO_4$.

Standardized solutions of enterochelin in ethyl acetate were prepared from stock 1 mM ferric-enterochelin. The pH of an aliquot of ferric-enterochelin solution was adjusted to approx. pH 1 with 2 M H_2SO_4 and the solution extracted twice with 1 vol. ethyl acetate. The ethyl acetate extracts were com-

bined and washed twice with 1 vol. 0.1 M phosphate buffer (pH 7.2). Following centrifugation, the ethyl acetate phase was removed and dried over anhydrous $MgSO_4$ and the concentration of enterochelin determined spectrophotometrically. Aqueous solutions of enterochelin were prepared by evaporating, over 0.1 M phosphate buffer (pH 7), appropriate volumes of standardized enterochelin/ethyl acetate solution. 2,3-Dihydroxybenzoylserine was prepared from 1 mM ferric-enterochelin as described previously [8].

Bacterial strains. E. coli K-12 mutants (AN194, AN273 (*fes*B), AN102 (*fep*), AN41 (*ent*E) have been described previously [1,5,9]. Strains were maintained on nutrient agar slopes containing 30 mM glucose/10 mM sodium citrate and were stored at 4°C.

Culture media, growth of cells and preparation of purified cell extract. Media and growth conditions for strains AN194 and AN41 have been described previously [11]. For growth of strain AN273, dipyridyl was replaced by 0.2 mM sodium citrate while, for strain AN102, dipyridyl was replaced by 0.2 mM $FeSO_4$. Purified cell extracts were prepared as described previously [11].

Gel column chromatography. Samples were applied to a 2 $cm^2 \times 90$ cm column of Sephadex G-150 which, unless otherwise specified, was equilibrated with 50 mM Tris · HCl (pH 8.0)/5 mM dithiothreitol. Samples were washed through the column at 4°C with the same buffer (flow rate 4.4 ml/h, 3-ml fractions).

DEAE-Sephadex column chromatography. Samples were dialysed against the starting buffer, 10 mM Tris · HCl (pH 8.0)/5 mM dithiothreitol/0.2 M NaCl, and applied to 4 $cm^2 \times 30$ cm columns of DEAE-Sephadex A-50 equilibrated with the same buffer. A linear gradient of 0.2—0.6 M NaCl in 1 l 10 mM Tris · HCl (pH 8.0)/5 mM dithiothreitol was applied without preliminary washing with initial buffer (20 ml/h, 10-ml fractions).

Assay for enterochelin esterase activity. The method used to measure activity of enterochelin esterase was similar to that described previously [4]. Unless otherwise stated, the reaction mixture contained (in a final volume of 0.3 ml): enzyme, 3 μmol dithiothreitol, 10 μmol Tris · HCl (pH 8.0) and 60 μmol of either enterochelin or ferric-enterochelin. After incubation at 37°C for either 15 min (enterochelin) or 60 min (ferric-enterochelin), the hydrolysis was stopped by the addition of 0.1 ml 1 M HCl. Enterochelin and hydrolysis products were extracted into 2.5 ml ethyl acetate. A 2 ml aliquot of the organic phase was extracted with 3 ml 0.1 M phosphate buffer (pH 7.2). Emulsions which formed during extractions were allowed to equilibrate for 20 min before centrifugation. Hydrolysis products, when present, were extracted into the phosphate buffer. Fluoresence associated with these products was measured on a Perkin-Elmer MPF-4 fluorescence spectrophotometer (activation, 333 nm; emission, 428 nm; uncorrected). Since the hydrolysis products all have similar fluorescence properties [4], levels of total hydrolysis products were determined, using standard curves prepared with, and expressed in units of 2,3-dihydroxybenzoylserine. Non-enzymatic hydrolysis of enterochelin was determined by omitting enzyme from reaction mixtures. In experiments aimed at clarifying the nature of the previously described hydrolase, enterochelin was used as substrate.

Assay for enterochelin synthetase (component E) activity. The method used

to measure activity of enterochelin synthetase (component E) has been described previously [11].

Estimation of protein. Protein was estimated by the method of Lowry et al. [13] with bovine serum albumin, fraction V, as standard.

Concentration and storage of enzyme fractions. When necessary, solutions of protein were concentrated in Diaflo ultrafiltration cells at 4°C using PM-10 membranes. Concentrated enzyme fractions were stored at −10°C.

Results

Relationship between enterochelin esterase and enterochelin hydrolase

In order to establish the relationship between enterochelin esterase and enterochelin hydrolase, samples of each material were prepared as described previously [4,6].

Enterochelin hydrolase activity was eluted from DEAE-Sephadex in a single peak at a position corresponding to 0.23 M NaCl on the salt gradient (Fig. 1A), and before the component E of enterochelin synthetase [11]. When purified cell extract prepared from strain AN273 (*fes*B) was fractionated on an identical DEAE-Sephadex column, no enterochelin hydrolase activity was detected (Fig. 1B). Strain AN273 is unable to hydrolyse ferric-enterochelin and has been reported by Langman et al. [5] to lack a functional B component of enterochelin esterase.

When partially purified enterochelin esterase was prepared in the presence of dithiothreitol by fractionating cell-free extract on Sephadex G-150, results markedly different from those reported previously [4] were obtained; a single

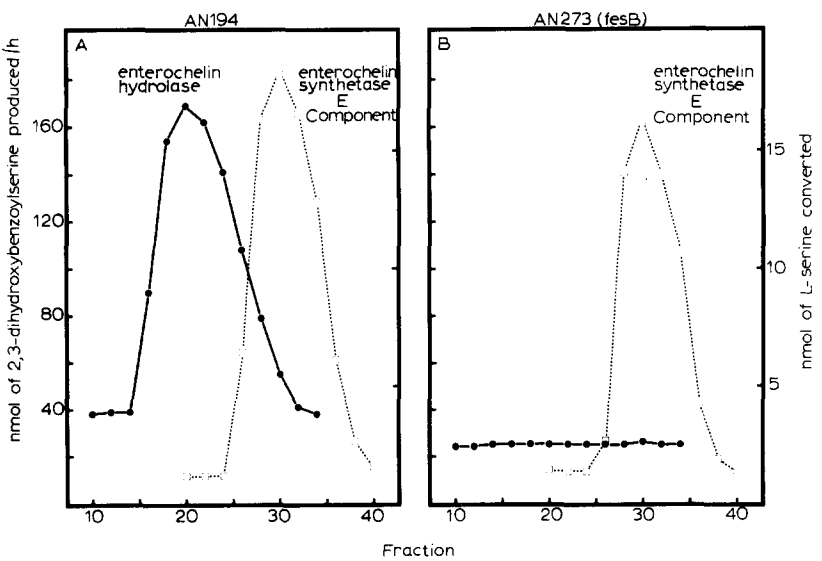

Fig. 1. Chromatography on DEAE-Sephadex of purified cell extract prepared from strain AN194 (frame A) and strain AN273 (frame B). Samples applied (10 ml) contained 20 mg/ml protein. Samples (100 μl) of fractions were assayed for enterochelin synthetase (component E) activity (□- - - - -□). Other samples (200 μl) were assayed for enterochelin hydrolase activity (●——●) using enterochelin as substrate. Conditions used during chromatography and assay were as described in Materials and Methods.

peak of activity was detected without any mixing of fractions (Fig. 2). The position of this peak corresponded to that of component B of enterochelin esterase. These results are consistent with the view that enterochelin hydrolase and component B of enterochelin esterase are the same entity. Furthermore support for this view was obtained when partially purified enterochelin hydrolase (Fig. 1A, fractions 15—25) was also passed through a column of Sephadex G-150. A single peak of enterochelin esterase activity was found at a position which was indistinguishable from that of component B of enterochelin esterase. Both enterochelin and ferric-enterochelin were hydrolysed (Fig. 3).

Substrate specificity of enterochelin esterase

Enterochelin esterase hydrolyses both enterochelin and ferric-enterochelin (Fig. 3). The relative rates at which the two substrates were hydrolysed by three different preparations of enterochelin esterase are shown in Table I. Under the assay conditions used, free enterochelin was hydrolysed approx. 2.5 times faster than was the enterochelin moiety of ferric-enterochelin.

Inhibition of enterochelin esterase activity by N-ethylmaleimide

The effects of *N*-ethylmaleimide and dithiothreitol on the activity of a partially purified preparation of enterochelin esterase were determined. More ferric-enterochelin was hydrolysed during the 60 min incubation period when dithiothreitol was present (82.9 nmol/h product), while *N*-ethylmaleimide inhibited enterochelin esterase activity (4.3 nmol/h product; compared to control of 69.1 nmol/h product). Activity was similarly inhibited by *N*-ethylmaleimide when enterochelin was used as substrate (unpublished data). It appears

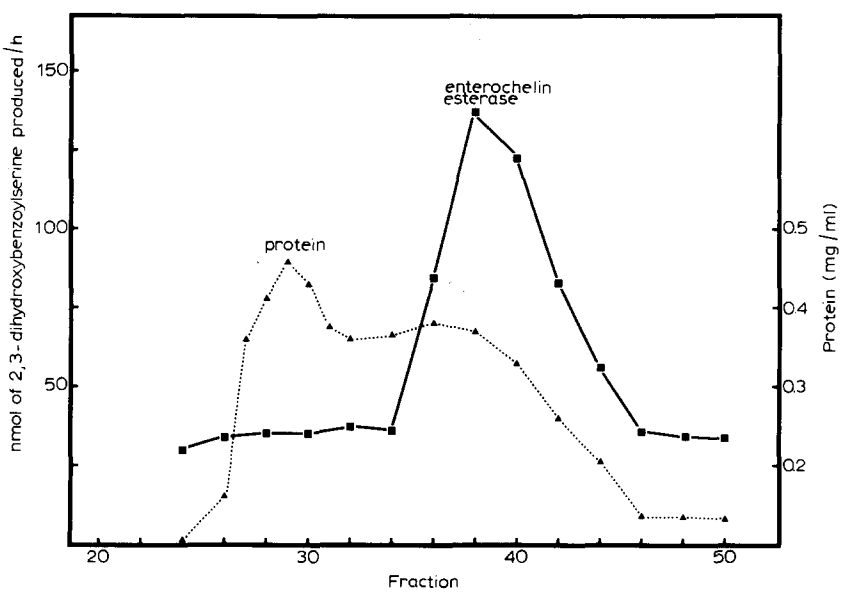

Fig. 2. Chromatography on Sephadex G-150 of a 2 ml sample of purified cell extract (20 mg/ml) prepared from strain AN194. Samples (100 μl) of fractions were assayed for enterochelin esterase activity (■———■) using ferric-enterochelin as substrate, or for protein (▲- - - - -▲). Conditions used during chromatography and assay were as described in Materials and Methods.

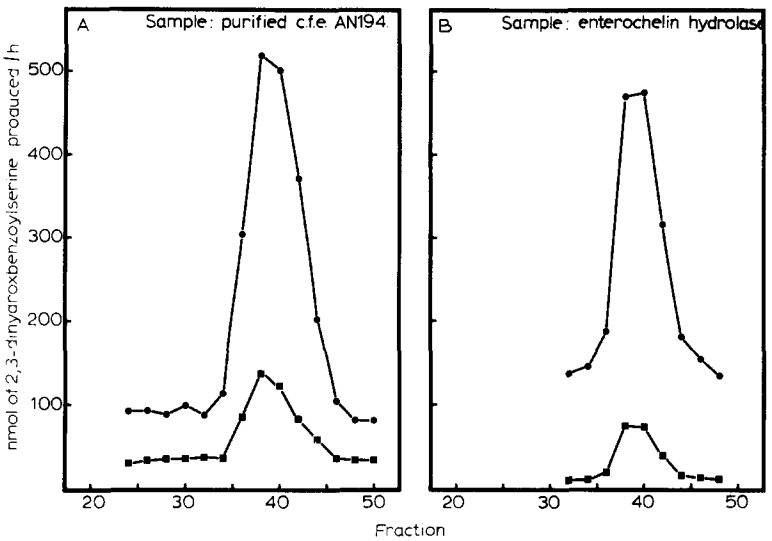

Fig. 3. Chromatography on Sephadex G-150 of purified cell extract prepared from strain AN194 (frame A), and of partially purified enterochelin hydrolase (frame B) obtained by pooling fractions 15—25 collected during chromatography on DEAE-Sephadex (Fig. 1A). Samples applied (2 ml) contained 20 mg/ml protein (AN194) or 3.7 mg/ml protein (hydrolase). Samples (100 μl) of fractions were assayed for enterochelin esterase activity using either enterochelin (●———●) or ferric-enterochelin (■———■) as substrate. Conditions used during chromatography and assay were as described in Materials and Methods.

that enterochelin esterase possesses at least one thiol group which is essential for activity.

Stability of enterochelin esterase

In order to study further the stability of enterochelin esterase, partially purified enzyme, free from dithiothreitol, was prepared by passing AN194 cell extract through a column of Sephadex G-150 equilibrated with 50 mM Tris ·

TABLE I

SUBSTRATE SPECIFICITY OF ENTEROCHELIN ESTERASE

Semi-purified enterochelin esterase was prepared by concentrating a mixture of fractions 15—25 obtained during chromatography of the purified cell extract on DEAE-Sephadex (Fig. 1A). The assay conditions were as described in Materials and Methods except that reaction mixtures containing ferric-enterochelin as substrate were incubated at 37°C for 15 min.

Source of enzyme	Amount of protein in reaction mixture (μg)	Activities of enterochelin esterase (nmol/h 2,3-dihydroxybenzoylserine produced)		Rate of hydrolysis of enterochelin / Rate of hydrolysis of ferric-enterochelin
		Enterochelin as substrate	Ferric-enterochelin as substrate	
AN194	175	88.0	39.6	2.2
Purified AN194 cell extract	105	235.2	86.8	2.7
Semi-purified enterochelin esterase	19	140.4	59.2	2.4

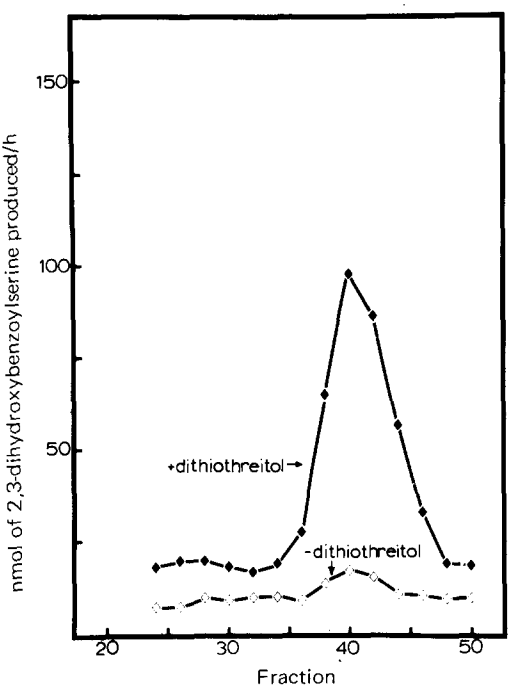

Fig. 4. Chromatography on Sephadex G-150, in the absence of dithiothreitol, of 2 ml purified cell extract (20 mg/ml protein) prepared from strain AN194. Samples (100 μl) of fractions were preincubated at 37°C for 10 min in the presence (♦———♦), or absence (◇———◇) of 10^{-2} M dithiothreitol before being assayed for enterochelin esterase using ferric-enterochelin as substrate. Conditions used during chromatography and assay were as described in Materials and Methods, except when dithiothreitol was omitted from reaction mixtures.

HCl (pH 8.0). When fractions were preincubated at 37°C for 10 min before being assayed for enterochelin esterase activity, little activity was detected (Fig. 4); these results resemble those reported previously by O'Brien et al. [4]. When dithiothreitol was included in reaction mixtures, a distinct peak of enterochelin esterase activity was observed (Fig. 4). In view of the full recovery of enterochelin esterase activity following chromatography at 4°C, it is evident that enterochelin esterase is stable at this temperature even in the absence of dithiothreitol. However it appears that, at 37°C, this substance prevents an otherwise considerable loss of activity. Further evidence for the stabilizing effect of dithiothreitol is shown in Fig. 5, where it is evident that activity was lost rapidly when enzyme was incubated at 37°C in the absence of both dithiothreitol and ferric-enterochelin. However little further loss of activity was observed following addition of substrate. This together with the observation that hydrolysis of ferric-enterochelin is linear throughout a 60 min incubation, even in the absence of dithiothreitol, indicates that the enzyme is stabilized in the presence of ferric-enterochelin. In other experiments (unpublished data), similar stabilization of activity was observed in the presence of enterochelin.

Stability of enterochelin esterase is also increased in the presence of various protein fractions. Mixtures of partially purified enterochelin esterase with either cell extract prepared from the mutant strain AN273 (*fes*B), or with material obtained from the void volume following gel chromatography (Fig. 4),

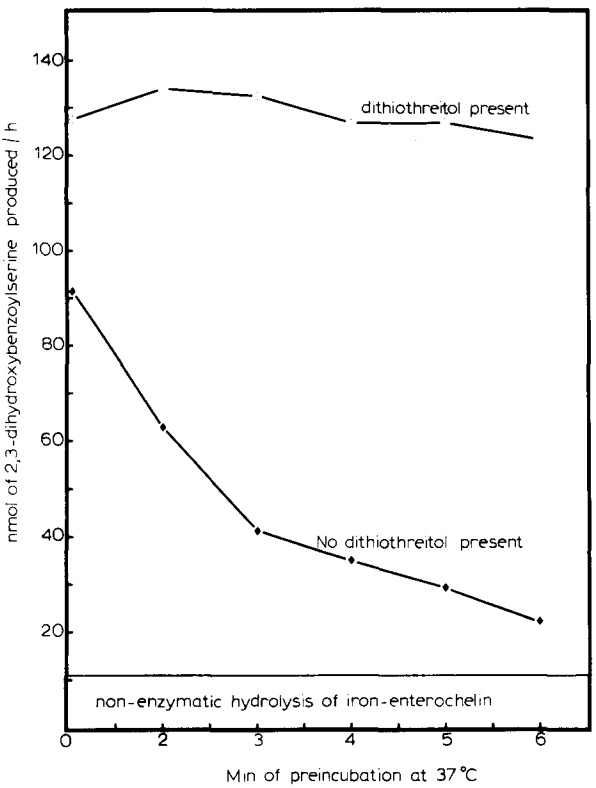

Fig. 5. Stabilization of enterochelin esterase by dithiothreitol during incubation at 37°C in 60 mM Tris · HCl (pH 8.0). Samples (100 μl) of semi-purified enterochelin esterase (fraction 40, Fig. 4) were incubated at 37°C in the presence (◇——◇) or absence (◆——◆) of 10^{-2} M dithiothreitol. After the intervals shown, substrate (ferric-enterochelin) was added and enterochelin esterase activity measured. Reactions were linear throughout the 60 min incubation. Conditions used during chromatography and assay were as described in Materials and Methods except when dithiothreitol was omitted from reaction mixtures.

TABLE II

STABILIZATION OF ENTEROCHELIN ESTERASE BY VARIOUS PROTEIN FRACTIONS AND BY DITHIOTHREITOL DURING A 10 MIN PREINCUBATION AT 37°C

Enterochelin esterase (33 μg protein) was obtained from fraction 40 following chromatography of purified AN194 cell extract on Sephadex G-150 (Fig. 4). A component was obtained by concentrating a mixture of fractions 26–30 (Fig. 4), and dithiothreitol was included where indicated at a final concentration of 10^{-2} M. Ferric-enterochelin was used as substrate. The assay conditions were as described in Materials and Methods except that the reaction mixtures did not contain dithiothreitol and were preincubated for 10 min at 37°C prior to the addition of substrate.

Addition to normal reaction mixture during preincubation	Amount of additional protein (μg) added to reaction mixture	Activity of enterochelin esterase following preincubation (nmol/h 2,3-dihydroxybenzoylserine produced)
Nil	—	9.1
AN273(*fes*B) cell extract	500	76.2
A component	500	41.3
Repressed AN194 cell extract	500	91.6
Bovine serum albumin, fraction V	500	66.4
Dithiothreitol	—	74.4

were much more active after 10 min preincubation in the absence of substrate than were samples of enzyme not mixed with other proteins (Table II). Neither the material from the void volume nor that from the mutant strain showed significant levels of activity when assayed separately under the same conditions. These results resemble those obtained by O'Brien et al. [4] and could be interpreted as indicating the presence of two complementary components. However a similar result was obtained when either repressed cell extract prepared from strain AN194 or bovine serum albumin was mixed with partially purified esterase (Table II). This stabilization by unrelated proteins indicates that the component A described previously is an artifact.

Discussion

The importance of enterochelin esterase in enterochelin-mediated transport of iron has been demonstrated previously [4,14]. We have now shown that the enzyme which hydrolyses the ester bonds of enterochelin chromatographs as a single component on Sephadex G-150 and corresponds to enterochelin hydrolase and component B of enterochelin esterase described previously. This enzyme, for which we suggest the continued use of the trivial name enterochelin esterase, is able to hydrolyse the ester bonds of enterochelin whether or not the molecule is complexed with iron. Under our assay conditions, free enterochelin is hydrolysed 2.5 times faster than enterochelin which is complexed with iron.

One possible explanation for the discrepancies between our results and those of Bryce and Brot [6] who suggested that ferric-enterochelin is not hydrolysed enzymatically, is based on the observation that non-enzymatic breakdown of ferric-enterochelin is slower than corresponding breakdown of enterochelin. Addition of excess ferric ion to a reaction mixture, in which enterochelin is being enzymatically hydrolysed, would be expected to result in a decrease in the overall rate at which breakdown products are formed. This could be interpreted as indicating inhibition of enzymatic breakdown if appropriate controls were not carried out. Whether this is in fact the reason for the discrepancies cannot be established with certainty, since complete details of the assay procedure used previously are not available.

Enterochelin esterase activity is very unstable at 37°C. However, the activity may be stabilized by the presence of dithiothreitol, ferric-enterochelin, enterochelin or a number of different proteins. It was presumably stabilization of the single component esterase by other unrelated proteins which prompted O'Brien et al. [4] to report the existence of a complementary protein factor (component A). Since enterochelin esterase activity is sensitive to *N*-ethylmaleimide, it seems likely that the enzyme contains at least one thiol group which is essential for activity. It may be that the stabilizing effect of other proteins results from "protection" of a reactive thiol group at the active site of the enzyme.

Uncertainties concerning the nature and substrate specificity of enterochelin esterase have now been resolved. The single-component enzyme is capable of hydrolysing the ester bonds of the powerful ligand of the ferric-enterochelin complex to produce weaker ligands from which the metal ion may be taken.

Acknowledgements

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