TWO INTERCONVERTIBLE FORMS OF A PURIFIED *E. COLI* ESTERASE SPECIFIC FOR APNE*

Ph. REGNIER, E. SACHE** and M. N. THANG
Institut de Biologie Physico-chimique, 13 rue P. et M. Curie, 75005 Paris, France and
***Institut Choay, 10 rue Morel, 92120 Montrouge, France*

Received 23 April 1974

1. Introduction

During the course of studies on proteolytic enzymes in *E. coli* ML 304 G [1], hydrolytic activity toward APNE – a conventional substrate for chymotrypsin [2] – was revealed in several cytoplasmic protein fractions separated on a DEAE-cellulose column.

One of these esterases, with a defined $R_m = 0.26$ in polyacrylamide gel electrophoresis, was studied in a fraction also possessing caseinolytic activity (ref. [1], fig. 5, band 4). The protease in this fraction was characterized and called protease C [3]. This communication describes the esterase which has been purified to homogeneity.

The enzyme is not the *E. coli* protease I, isolated by Pacaud and Uriel [4]; it is devoid of hydrolytic activity toward both casein and *E. coli* polynucleotide phosphorylase; it appears to be an oligomeric enzyme with interesting molecular properties.

2. Materials and methods

The products used for the present work originate as follows:

– Casein, acetylphenylalanylnapthylester (APNE), Schwarz-Mann Research Laboratories (USA);

– ^{125}I, C.E.A., Saclay (France);

– Tetrazotized-di-O anisidine Zinc double salt, Fluka (Switzerland);

– Acrylamide, bis-acrylamide, Temed, Coomassie blue, Bromophenol blue, Canalco Europe (The Netherlands).

2.1. *Preparation of acellular extracts*

E. coli ML 304 G auxotroph for tryptophan was grown on the following minimum medium; for a final volume of 1 liter: KH_2PO_4, 13.6 g; $(NH_4)_2SO_4$, 2.0 g; $MgSO_4$, 0.1 g; $FeSO_4$, 0.5 mg, glucose, 2.0 g, and tryptophan, 40 mg. The pH is adjusted to 7.4 with NaOH. Bacteria are collected during the exponential phase ($A_{420nm} = 3$), washed twice with 0.01 M Tris–HCl buffer (pH 7.5), and the acellular extracts are then prepared by sonication.

One g of wet bacteria is suspended in 3 ml of 0.01 M Tris–HCl (pH 7.5) and sonicated three times for 2 min by fractions of 1 ml in an MSE ultra-sound generator, 100 W; the cellular debris is eliminated by two centrifugations of 30 min at 30 000 *g*.

2.2. *Assays for esterolytic activity*

The esterolytic activity was determined using the following compounds:

– APNE (hydrolytic activity is determined in situ after electrophoretic migration of the proteins on acrylamide gel. The method has been described by Pacault and Uriel for agarose-acrylamide gels [4]).

– *N*-Benzoyl-L-arginine ethyl ester (BAEE); tosyl-L-arginine methyl ester (TAME), *N*-acetyl-L-lysine methyl ester (ALME), *N*-acetyl(alanine)^3 methyl ester (AAla^{3}ME), and *N*-acetyl-L-tyrosine methyl ester (ATEE); the assay medium (final volume 1.1 ml) contains: 20 mM $CaCl_2$, and 20 mM substrate in 1.5 mM borate buffer (pH 8.0); the activity was deter-

* APNE = acetylphenylalanylnaphthylester.

mined in a pH-stat with NaOH 10 mM.

– *N*-Benzoyl-DL-arginine paranitroanilide (BAPNA): the method is that of Ganrot [5]. The incubation medium contains: 37 mM Tris–HCl (pH 8.2); 7.5 mM $CaCl_2$; 1.1 mM BAPNA.

– Carbobenzoxy-L-tyrosine paranitrophenyl ester (CBZTyr): the incubation medium (3 ml) contains 0.1 M Tris–HCl (pH 8.2); 2 mM $CaCl_2$; 0.05 mM CBZTyr; 3% acetone; the O.D. increase is followed at 400 nm.

Amidase activity was also assayed, using L-leucyl-β-naphthylamide as substrate, according to Arst et al. [6].

2.3. *Assays for proteolytic activity*

– With polynucleotide phosphorylase (PNPase) as substrate, the incubation medium (100 μl) contains: 1 μg PNPase; 0.01 M Tris–HCl (pH 9) and the enzymatic extract. After incubation at 37°C, the mixture is subjected to electrophoresis on 7.5% acrylamide gels [7]. PNPase is revealed in the gel in situ [8].

– ^{125}I-labelled casein: the method used has been previously described [1].

2.4. *Acrylamide gels electrophoresis and protein elution*

The molecular weights of the native enzymes was determined by the technique of Hedrick and Smith [9]; the buffers used were those described by Davis [7].

The molecular weights of the denatured enzymes was determined according to Weber and Osborn [10]; the acrylamide concentration of the gels was 5%.

The technique used for protein elution from polyacrylamide gel is derived from that described by Thang et al. [11]. After electrophoresis of the extracts [7], one gel is stained for esterase activity. In the other gels a slice is removed at the level of the active band. These slices are introduced into a glass tube; the lower part of the tube, where the proteins are to be eluted, is filled up with the electrophoresis buffer and closed with a piece of dialysis bag; an intensity of 6 mA is applied for 30 min. The part of the tube between the slice of gel and the porous membrane, which now contains the proteins eluted from the gel, is immerged, membrane first, into 10 mM Tris buffer (pH 7.5), with or without β-mercaptoethanol as might be the case. Dialysis is performed during a minimum of 4 hr. The membrane is then pierced and the content of the elution chamber is collected. The elution chamber, (i.e. the volume contained between the slice of gel and the dialysis membrane) has a volume between 40 and 200 μl according to the experiments.

3. Results

3.1. *Visualization of APNE esterase*

As described in Materials and methods, the activity toward APNE can be revealed in situ on polyacrylamide gel after a suitable electrophoretic run of an acellular extract. Three cases were frequently observed:

(1) When the extract was analyzed directly immediately after sonication, APNE hydrolysis resulted in one major band with $R_m = 0.26$;

(2) When the extract was dialyzed at 4°C against 10 mM Tris–HCl buffer, pH 7.5, before gel electrophoresis, two stained bands were obtained, one with the same R_m as above, and the other with a higher mobility;

(3) When the dialysis was prolonged (usually for 18 hr) only the faster-migrating band was observed.

For presentation commodity we will call the slower migrating protein, also possessing APNE hydrolyzing activity, APNE-esterase form A, and the faster migrating protein, APNE-esterase form B. These enzymes might be two distinct proteins having the same activity toward APNE, one being inactivated under the conditions used for dialysis and the other being activated. Alternatively, A and B might be the same enzyme assuming different forms modulated by dialysis conditions.

3.2. *Interconversion of the two forms*

Two characteristics of forms A and B suggested that the phenomenon observed might result from an interconversion of the same enzyme. First, the presence of a thiol, (e.g. β-mercaptoethanol) prevents the appearance of form B, even after prolonged dialysis. Second, when form B was obtained by dialysis, it was possible to revert to form A by a 1 hr incubation at 25°C in the presence of β-mercaptoethanol. Thus, it seems likely that form B is a molecular species of the APNE esterase A oxidized under dialysis

conditions, and that the oxidized form, i.e. B, can be quantitatively converted into the initial enzyme form, i.e. A.

On the other hand, oxidation of this esterase was also inhibited by EDTA, suggesting that metal ion-catalyzed oxidation might be involved.

3.3. *Purification of the APNE esterase*

Based on this interconvertibility, the esterase can easily be purified to homogeneity in small quantities, using only two electrophoretic runs on polyacrylamide gels. Actually, we first separated on the gels an extract dialyzed for 18 hr against Tris–HCl buffer, to ascertain the esterase is quantitatively converted into form B. After electrophoresis, one gel was assayed for APNE hydrolysis in order to localize the enzyme; by comparing with this control gel, one could easily pick up the slice, in the other gels, containing APNE esterase activity. Each of these slices was introduced in a glass cylinder for microelution as described in Materials and methods. The eluted proteins were dialyzed against 10 mM Tris–HCl buffer, pH 7.5, with 10 mM β-mercaptoethanol to convert the esterase form B into form A. The dialyzed proteins were directly applied onto a series of polyacrylamide gels for a second electrophoretic run. The esterase was localized, as previously, on a control gel which showed a purple band at the place of form A, and was thus separated from other eluted proteins which migrated to the position of form B (fig. 1). The esterase, now in form A, was again eluted from the gel slices and dialyzed as previously. Electrophoresis of the eluted proteins showed a single band that could be stained with Coomassie blue, and revealed by its APNE hydrolizing activity (fig. 1).

3.4. *Molecular weight and subunits of APNE esterase*

The molecular weights of the esterase, both in forms A and B, were determined directly with acellular extracts by the procedure of Hedrick and Smith [9]. Three cases have been examined: (1) the sonic acellular extract without treatment for form A; (2)

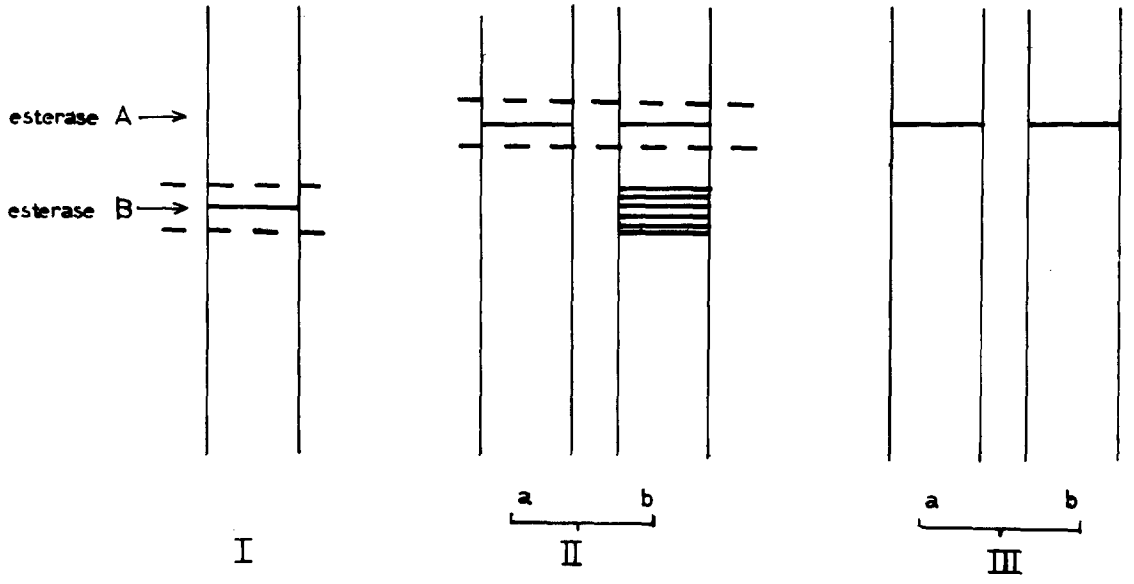

Fig. 1. Gel I: After electrophoresis on a polyacrylamide gel (length 6 cm, ϕ 6 mm) of 500 µl of proteins from a dialyzed crude extract, the esterase activity was revealed in situ by hydrolysis of APNE; Gel II: Aliquots of proteins eluted from slices of gel I cut out at the level of the activity band were dialyzed against 10 mM Tris–HCl, pH 7.5, and 10 mM β-mercaptoethanol and separated by electrophoresis. – Esterase activity: gel II_a. – Proteins stained with Coomassie blue: gel II_b. (The proteins migrating at the level of esterase B are schematically represented by parallel lines. The dotted lines schematically correspond to the cutting out of the gel slices on both sides of the esterase activity bands. Gel III: Aliquots of proteins eluted from slices of gel II_a cut out at the level of the activity band were separated by electrophoresis. – Esterase activity on APNE: gel III_a. – Proteins stained with Coomassie blue: gel III_b.

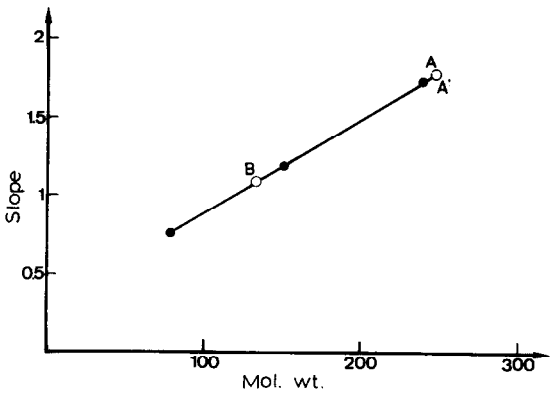

Fig. 2. Evaluation of molecular weights of the different esterase forms. 500 μg of crude extract (esterase A), of dialyzed crude extract (esterase B) and of dialyzed crude extract preincubated in the presence of β-mercaptoethanol (esterase A') were each separated by electrophoresis on gels containing various acrylamide concentrations in order to determine their molecular weights. The markers used were: alkaline phosphatase (M.W. 80 000), alcohol dehydrogenase (M.W. 150 000) and pyruvate kinase (M.W. 237 000).

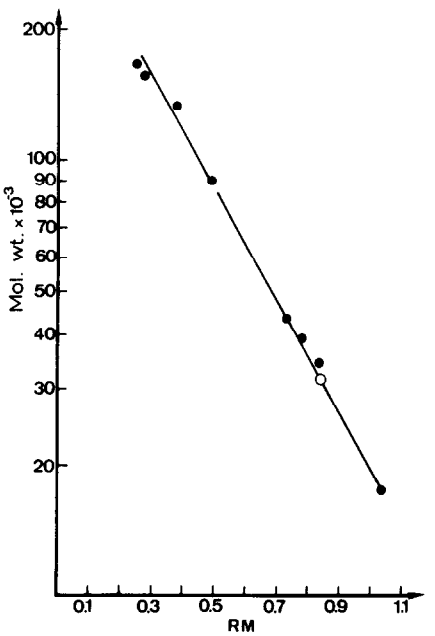

Fig. 3. Study of the esterase molecular weight in denaturing medium. The molecular weight was estimated according to Weber and Osborn [10]. The proteins were revealed by staining with Coomassie blue. The markers used were: RNA-polymerase (M.W.: β 165 000; β' 155 000; σ 90 000; α 39 000); β-galactosidase (M.W. 135 000); ovalbumin (M.W. 43 000); aspartate transcarbaonylase (M.W. 34 000 and 17 000).

the dialyzed extract for form B; (3) reversion of the B form by incubation of the dialyzed extract in the presence of β-mercaptoethanol. Fig. 2 shows that form A has a molecular weight of about 230 000, form B about 120 000 and the form obtained from form B by β-mercaptoethanol incubation, around 230 000. These figures suggest that form B is a dissociated product from form A, and that the reversed form seems to be the reassociation of two molecules of B in order to yield form A.

The subunit composition of the esterase was also studied by electrophoretic analysis of the purified enzyme. The purified esterase was first dissociated at 100°C in 8 M urea containing 0.1% SDS and 0.1 M β-mercaptoethanol before electrophoresis on SDS-polyacrylamide gel. A single protein band was obtained, with a molecular weight of about 32 000 (fig. 3).

3.5. *Hydrolytic and proteolytic activities*

The purified esterase has no detectable hydrolytic activity on ATEE, BAEE, TAME, ALME, BAPNA, AAle[3]ME, and leucyl-β-naphthylamide. Its substrate specificity seems to be very narrow, although some of the assays used here are not highly sensitive.

Nor has the purified esterase any detectable proteolytic activity on ^{125}I-labelled casein. Polynucleotide phosphorylase from *E. coli*, an enzyme which is very sensitive to proteolytic cleavage, either by trypsin or by *E. Coli* endogenous protease, is not at all degraded by the esterase.

4. Discussion

By a very simple procedure we have purified an *E. coli* esterase almost to homogeneity, on an analytical scale. The enzyme has a narrow specificity for an ester substrate, since among a variety of esters, only hydrolytic activity towards APNE was detected. Due to this specificity, the enzyme may thus be considered as a chymotrypsin-like esterase. Although the esterase cannot hydrolize casein or *E. coli* polynucleotide phosphorylase this does not exclude the possibility of it being able to cleave peptide bonds. The proteolytic activity might depend on some structural elements of the protein.

One interesting aspect of this APNE esterase is the

interconvertibility of two molecular forms that can easily be seen on polyacrylamide gels; both these forms (A and B), are active. The molecular weights of form A and form B, estimated as 230 000 and 120 000 respectively, suggest a reversible dissociation–association of the enzyme with a possible relation of: $A \rightleftharpoons 2B$. Anyhow, it is noteworthy that the enzyme can be split into a molecular species of half the molecular weight of the original enzyme and still retaining its activity. The mechanism of this reversible dissociation is not as yet understood.

The esterase is an oligomeric enzyme. In fact, analysis of the purified enzyme by electrophoresis in dissociating polyacrylamide gel in the presence of SDS shows one protein band having an estimated molecular weight of 32 000. Thus, it seems likely that APNE-esterase is composed of only one kind of polypeptide chain. We assume an uncertainty of ± 10% to ± 15% for all our measurements of molecular weights, for the subunit as well as for the A and B forms; it is therefore premature to assign a subunit composition to the enzyme before the molecular weights have been determined with more precision.

Acknowledgements

This work was supported by the Centre National de la Recherche Scientifique (G.R.No. 18) and by Délégation Générale à la Recherche Scientifique et Technique (Convention No. 72.7.0581).

We are very grateful to Dr. Pommier from Unité de Recherche sur la Glande Thyroïde et la Régulation Hormonale, INSERM Laboratory in Bicêtre for the preparation of ^{125}I-labelled casein.

References

[1] Regnier, Ph. and Thang, M. N. (1972) Biochimie 54, 1227–1236.
[2] Ravin, H. A., Bernstein, P. and Seligman, A. M. (1954) J. Biol. Chem. 208, 1–5.
[3] Regnier, Ph. and Thang, M. N. (1973) Compt. Rendu. 277 D, 2817–2820.
[4] Pacaud, M. and Uriel, J. (1971) Eur. J. Biochem. 23, 425–442.
[5] Ganrot, P. O. (1966) Act. Chem. Scand. 20, 175–182.
[6] Arst, H. E., Manning, R. T. and Delp, M. (1959) Amer. J. Med. Sci. 238, 598–609.
[7] Davis, B. J. (1964) Ann. N. Y. Acad. Sci. 121, 404–427.
[8] Thang, M. N., Thang, D. C. and Leautey, J. (1967) Compt. Rendu. 265 D, 1823–1825.
[9] Hedrick, J. L. and Smith, A. J. (1968) Arch. Biochem. Biophys. 126, 155–164.
[10] Weber, K. and Osborn, M. (1969) J. Biol. Chem. 244, 4406–4412.
[11] Thang, M. N., Dondon, L. and Godefroy-Colburn, Th. (1971) Biochimie 53, 291–302.