Biochimica et Biophysica Acta, 568 (1979) 395—407
© Elsevier/North-Holland Biomedical Press

BBA 68756

THE SOLUBILISATION OF THE MEMBRANE-BOUND D-ALANYL-D-ALANINE CARBOXYPEPTIDASE OF *BACILLUS COAGULANS* NCIB 9365

HAMISH A.I. McARTHUR * and PETER E. REYNOLDS

Sub-Department of Chemical Microbiology, Department of Biochemistry, University of Cambridge, Tennis Court Road, Cambridge CB2 1QW (U.K.)

(Received November 27th, 1978)

Key words: Carboxypeptidase; Solubilization; Detergent; Chaotropic agent; Alanyl-alanine

Summary

Protoplast membranes and the particulate D,D-carboxypeptidase of *Bacillus coagulans* NCIB 9365 were extremely resistant to disruption by either detergents or urea. A combination of urea and the non-ionic detergent Genapol X-100 was required to achieve a significant solubilisation of membrane protein and D,D-carboxypeptidase in an active form; the pH optimum for this treatment was pH 7.5. Solubilisation of the enzyme was accompanied by a two-fold enhancement of activity. Kinetic results indicated that the enhancement may be due to an alteration in the conformation of the enzyme following disruption of membrane structure.

Introduction

The D-alanyl-D-alanine carboxypeptidase (UDP-*N*-acetylmuramoyl-tetrapeptidyl-D-alanine alanine-hydrolase, EC 3.4.12.6) of *Bacillus coagulans*, as with that of most other microorganisms, is located exclusively in the cytoplasmic membrane of the cell [1—5]; although in some instances this enzyme may be found additionally in the cytoplasm [6] or excreted into the growth medium [7].

In order to study the D,D-carboxypeptidase in greater detail it is desirable to obtain a purified enzyme preparation; the first step towards which is the solubilisation of the membrane-bound enzyme. Several procedures have been

* To whom correspondence should be addressed at (present address): Microbiological Chemistry Research Laboratory, The University, Newcastle upon Tyne, NE1 7RU, U.K.

employed to remove D,D-carboxypeptidase from its particulate environment. Thus, up to 30% of the D,D-carboxypeptidase could be released from membranes of *Proteus mirabilis* [4] by osmotic shock techniques, while the D,D-carboxypeptidase from *Escherichia coli* B [8] was released from freeze-dried cells by resuspension in distilled water. Sonication has also been used to solubilise the particulate enzyme of *E. coli* B[9]; however, in most instances procedures have been adopted which disrupt the structural integrity of the membrane.

Although the solubilisation of 70% of the particulate D,D-carboxypeptidase of *B. stearothermophilus* has been achieved with *n*-butanol [10], detergents have been the primary method. Non-ionic detergents have been most widely employed [4,10—15], but the cationic detergent cetyltrimethylammonium bromide was also effective [10,16]. Urea, acting as a chaotropic agent [17], has also been used to obtain the solubilisation of the membrane-bound D,D-carboxypeptidase/transpeptidase of *Streptomyces* R61 [7], although the solubilised enzyme was unstable and the extraction non-reproducible [16].

The properties of the membrane-bound D,D-carboxypeptidase of *B. coagulans* NCIB 9365 have been established previously [1]; the purpose of the present paper is to report studies on the solubilisation of the particulate enzyme.

Materials and methods

Enzyme preparation and assay

Particulate enzyme was prepared in the form of lysed protoplast membranes as described previously [1]. Standard incubation mixtures contained (final concentrations): 0.025 M sodium acetate-acetic acid (pH 4.9); 0.015 M $MnCl_2$; 0.002 M UDP-MurNAc-L-Ala-D-Glu-*meso*-Dap-D-[^{14}C]Ala-D-[^{14}C]Ala and enzyme source in a final volume of 25 μl, and were assayed at 37° C for 20 min.

Substrates, UDP-MurNAc-L-Ala-D-Glu-*meso*-Dap-D-[^{14}C]Ala-D-[^{14}C]Ala, L-Ala-D-Glu-*meso*-Dap-D-[^{14}C]Ala-D-[^{14}C]Ala and UDP-MurNAc-L-Ala-D-Glu-*meso*-Dap-D-Ala-D-Ala were prepared, and D,D-carboxypeptidase activity was assayed as described before [1].

Enzyme activities are defined in terms of International Units (U) or milliunits wherever possible; one unit is equivalent to the liberation of 1 μmol D-alanine from UDP-MurNAc-L-Ala-D-Glu-*meso*-Dap-D-Ala-D-Ala.

Estimation of the degree of solubilisation

The degree of solubilisation may be expressed in terms of the original enzymatic activity present in untreated membranes, but certain compounds such as detergents may cause considerable enhancement or inhibition of enzymatic activity. Thus when this parameter is employed, the 'solubilisation' value obtained is the product of the interaction of two components: (a) the actual degree of solubilisation afforded; and (b) the effect of the solubilising conditions on enzymatic activity. This latter component may be circumvented partially by expressing results in terms of the distribution of enzyme between the pellet and supernatant fractions after centrifugation of the extracted preparation. However, following detergent extraction, the high-speed centrifugation required to achieve the criterion of solubility (see below) results in the sedi-

mentation of a residual pellet which proved difficult to resuspend as an homogeneous solution. Hence the reproducibility of assay of supposedly identical aliquots from such preparations was consistently less than 80%. This difficulty may be obviated by estimating the total enzymic activity of a treated preparation prior to centrifugation. Thus, following extraction of protoplast membranes under the appropriate conditions, a sample of this fraction (treated control) was retained for estimation of enzymic activity, after dialysis whenever required. The remainder of the solution was then subjected to centrifugation; the supernatant (soluble fraction) was decanted and enzyme assays were performed.

The degree of solubilisation was then expressed as:

$$\frac{\text{total soluble activity}}{\text{total activity treated preparation}}$$

The reproducibility of this method proved to be at least 90% for duplicate samples either from the same preparation or from membrane fractions extracted under similar conditions. This method therefore gives some estimation of the true degree of solubilisation of enzyme activity in the absence of any stimulatory or inhibitory effects of the extraction procedure.

However, for purification purposes the maximum release of enzyme in an active form is also important. This may be illustrated conveniently by expressing the yield of solubilised enzymatic activity as a percentage of the total activity of the untreated membrane preparation. Hence in each instance the degree of solubilisation was expressed relative to these two parameters.

Protein estimation

Protein was determined by the method of Lowry et al. [18] with 1% SDS in the stock solutions to prevent precipitate formation during assay in the presence of detergents [12].

Chemicals

Brij 35 and 58 were obtained from Honeywill Atlas, Carshalton, Surrey, U.K.; Tween 20, 60 and 80, and Span 85 from Koch-Light Laboratories (Colnbrook, Bucks., U.K.). Nonidet P40, Triton X-100, cetyltrimethylammonium bromide and sodium dodecyl sulphate were from British Drug Houses (Poole, Dorset, U.K.). Genapol X-80, X-100 and ZDM-090 were kindly provided by Farbwerke Hoechst AG (Frankfurt/Main-Hoechst, F.R.G.), and sodium deoxycholate was from Hopkin and Williams (Romford, Essex, U.K.). Phospholipase C (EC 3.1.4.3) was obtained from the Boehringer Corp., (Lewes, Sussex, U.K.).

Results

Sonication

It has been reported [9] that the particulate D,D-carboxypeptidase of *E. coli* B can be 'solubilised' by sonication. Following sonic disruption of *B. coagulans* for 5 min, the effect of differential centrifugation on the distribution of D,D-

carboxypeptidase, and endopeptidase (an enzyme catalysing the hydrolysis of D-Ala-D-Ala from UDP-MurNAc-L-Ala-D-Glu-*meso*-Dap-D-Ala-D-Ala [1]) between particulate and supernatant fractions, was, therefore, investigated.

After centrifugation at 38 000 × *g* for 40 min, virtually all the D,D-carboxypeptidase was found in the supernatant fraction, but with increased centrifugal force for longer periods, a greater proportion of the enzyme was found in the particulate fraction. Thus under the maximum centrifugation employed (225 000 × *g* for 2 h), only 12% of the D,D-carboxypeptidase was 'soluble'. It is apparent that the membrane-bound enzyme was not removed from its particulate environment by sonication, but that this procedure merely served to fragment the membrane into particles which were sedimented only at high centrifugal forces. This was confirmed by the fact that the endopeptidase, which is located in the cytoplasm of the cell [1] and hence is a 'soluble' enzyme, was not sedimented by any of the centrifugation conditions.

For the purpose of this investigation a protein was considered to have been solubilised if it did not sediment after centrifugation at 225 000 × *g* for 2 h.

As well as sonication, osmotic shock of membranes with low-ionic-strength solutions of Tris-HCl failed to release any D,D-carboxypeptidase activity. Thus, in order to solubilise the carboxypeptidase, procedures were employed which disrupt membrane structure.

n-Butanol

Treatment of membranes with *n*-butanol according to the procedure of Barnett [10] caused an inactivation of D,D-carboxypeptidase activity which was not restored by dialysis. Although this procedure has been effective in solubilising the D,D-carboxypeptidase of *B. stearothermophilus*, *n*-butanol was found to inactivate also the D,D-carboxypeptidases of *B. megaterium* (Reynolds, P.E., unpublished results) and *P. mirabilis* (Martin, H.H., unpublished results), while solubilisation of the particulate transpeptidase/carboxypeptidase of *Streptomyces* R61 with *n*-butanol lacked reproducibility and yielded an unstable enzyme preparation [16].

Detergent solubilisation

The effect of various detergents on the activity of particulate D,D-carboxypeptidase was investigated by their incorporation at a concentration of 0.5% (w/v) directly into incubation mixtures (Table I, Detergent effect). Of the non-ionic detergents tested, the Genapol series, Triton X-100 and Nonidet P40, produced a very marked enhancement of enzyme activity. The other non-ionic detergents did not demonstrate this enhancement and had little effect on the enzyme. The cationic cetyltrimethylammonium bromide and both anionic detergents inhibited enzyme activity to a considerable extent; this was most pronounced with SDS.

The ability of several detergents to 'solubilise' enzymatic activity and membrane protein is also shown in Table I. With the exception of SDS, none of the detergents released more than 30% of either enzyme activity or membrane protein. Although SDS solubilised almost 90% of the protein, as observed above, the detergent inactivated the enzyme; the activity was not restored after extensive dialysis. In no instance was the enzyme released selectively; hence

TABLE I

DETERGENT SOLUBILISATION OF PARTICULATE D,D-CARBOXYPEPTIDASE

2-ml samples of particulate enzyme (12 mg protein) in 0.05 M Tris-HCl (pH 7.2) were treated with 0.5 ml of the appropriate detergent (10% solution, w/v) in the above buffer, at 4°C for 30 min. After extraction, a 0.5 ml aliquot of each solution (treated control) was retained, and the remainder centrifuged at 225 000 × *g* for 2 h. The degree of solubilisation of enzyme activity was determined relative to the total activity of the treated preparation. The yield of soluble enzyme was estimated relative to the total enzyme activity of the untreated membranes (100%). The effect of the detergents on enzyme activity was determined by the assay of particulate enzyme in the presence of 0.5% (w/v) of the appropriate detergent (enzyme activity without detergent = 100%). HLB, hydrophilic-lipophilic balance.

Detergent	HLB	Detergent Effect (%)	Solubilisation (%)		Yield (%)
			Protein	Enzyme	
Genapol X-80	12.6 *	218	30	22	48
Genapol X-100	13.8 *	232	28	24	56
Genapol ZDM-090	—	214	21	25	54
Triton X-100	13.5	221	27	20	44
Nonidet P40	13.1	237	22	24	57
Tween 20	16.7	87	11	7	6
Tween 60	15.6	91	8	7	6
Tween 80	15.0	110	9	15	17
Brij 35	16.9	100	18	14	14
Brij 58	15.7	100	10	8	8
Span 85	1.8	92	7	6	6
Cetyltrimethylammonium bromide	—	31	15	9	2
Sodium deoxycholate	—	24	10	11	2
Sodium dodecyl sulphate	—	0	87	0	0

* Calculated [39].

solubilisation of protein was equivalent to that of the enzyme activity. Those detergents which gave the greatest enhancement of enzymatic activity also achieved the greatest degree of solubilisation.

Urea solubilisation

Membranes were treated with various concentrations of urea, and the degree of solubilisation of protein and enzymatic activity were estimated (Fig. 1). Increasing concentrations of urea resulted in a greater solubilisation of mem-membrane protein. The percentage of solubilisation of enzyme activity was also found to increase with urea concentration; again, release of enzyme was non-specific. Treatment of membranes with urea was not accompanied by any enhancement of enzyme activity; rather, considerable irreversible enzyme inactivation occurred at concentrations of urea greater than 3 M; thus treatment of membranes with 5 M urea resulted in a 90% loss of activity, in comparison with the untreated membranes. Therefore, although more of the D,D-carboxypeptidase was solubilised by 5 M than 3 M urea, much less of the enzyme was released in an active form. However, even at the concentration of urea which resulted in the maximal release of active enzyme, less than 25% of the activity of untreated membranes could be released.

Chaotropic agent-detergent solubilisation

Although the attempted solubilisation of the D,D-carboxypeptidase with

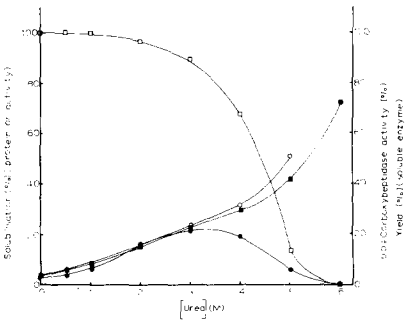


Fig. 1. Solubilisation of membrane-bound D,D-carboxypeptidase by urea. Protoplast membranes (4. 0 ml; 3.6 mg protein/ml) resuspended in 0.05 M Tris-HCl (pH 7.5) were treated with 9 M urea to achieve the final concentration indicated. and the solutions (final volume 12 ml) were stirred at 37°C for 15 min. Samples of each solution (treated controls) were retained and the remainder centrifuged at 225 000 × *g* for 120 min. The soluble fractions and treated controls were dialysed extensively against 0.05 M Tris-HCl (pH 7.5) prior to assay. ■, percent solubilisation of protein; ○, percent solubilisation of enzyme activity relative to the total enzyme activity of urea-treated preparations. Also shown are the yield of active soluble enzyme (●) estimated relative to the total activity present in the untreated membranes (100%), and the D,D-carboxypeptidase activity of the urea-treated controls (□) also relative to the activity of untreated membranes.

either detergents or urea alone was relatively unsuccessful, pilot experiments indicated that a large percentage of the enzymic activity could be released from protoplast membranes by a combination of these two treatments.

a. Nature of detergent. Protoplast membranes were treated with urea at a final concentration of 3 M at 37°C for 15 min. The appropriate detergent (Genapol X-80, X-100, ZDM 090, Triton X-100 or Nonidet P40) was added at a final concentration of 2% (w/v), and incubation was carried out at 4°C for a further 15 min. Following centrifugation, the degree of solubilisation was estimated. In conjunction with 3 M urea, any of the non-ionic surfactants solubilised more than 70% of the enzyme, with a resultant yield of 150% of the enzyme activity of untreated membranes, and 80% of the membrane protein. This was more than twice the value obtained with either of these procedures alone. However, there was little significant difference in the degree of solubilisation obtained between any of the detergents tested.

The Genapol series, unlike Triton X-100 and Nonidet P40, exhibit little absorbance of ultra-violet light at the concentration employed, a property of considerable importance during the monitoring of protein by the method of Warburg and Christian [19]. Genapol X-100 was available in a more refined form than either X-80 and ZDM-090 and hence was used in all further experiments

b. Nature of the chaotropic agent. The degree of solubilisation achieved by various chaotropic agents in conjunction with Genapol X-100 is shown in Table II. NaSCN, $NaClO_4$ and guanidine-HCl facilitated a greater degree of solubilisation of membrane protein than did equivalent concentrations of urea, but such efficient extraction was accompanied by an irreversible inactivation of the D,D-carboxypeptidase. This was most pronounced with SCN^-, although considerable inactivation also occurred with ClO_4^- and guanidine-HCl. The degree of inactivation of these alternative chaotropic agents was such that although urea

TABLE II

THE EFFECT OF VARIOUS CHAOTROPIC AGENTS IN THE PRESENCE OF GENAPOL X-100 ON THE SOLUBILISATION OF PARTICULATE ENZYME

Protoplast membranes (3 ml, 4.8 mg protein/ml) were extracted with the appropriate chaotropic agent (37°C, 15 min) and Genapol X-100 (4°C, 15 min) at final concentrations of 2 M and 2% (w/v), respectively, in a total volume of 5.3 ml. 1 ml of each solution was retained (treated control) and the remainder centrifuged at 225 000 × *g* for 2 h. The soluble fractions and treated controls were dialysed exhaustively against 0.05 M Tris-HCl (pH 7.5) prior to the estimation of enzyme activity. The degree of enzyme solubilisation was estimated relative to the total enzyme activity of the treated preparation. The yield of soluble enzyme was estimated relative to the total activity present in untreated membranes (100%).

Chaotropic agent	Soluble protein (mg)	Enzymatic activity (mU)		Solubilisation (%)		Yield (%)
		Treated preparation	Soluble	Protein	Enzyme	
None	3.0	224	51.5	21	23	54
Urea	7.5	236	136.0	52	58	142
NaSCN	14.8	14.0	13.6	103	97	14
$NaClO_4$	13.2	27.0	26.2	92	97	27
Guanidine-HCl	13.5	75	74	94	99	77
	Total protein (mg)	Enzymatic activity (mU)				
Untreated membranes	14.4	96 (100% yield)				

accomplished the solubilisation of less than 60% of the membrane protein, the yield of active enzyme was almost ten-fold, six-fold and two-fold greater than with SCN^-, ClO_4^- and guanidine-HCl respectively.

c. Optimal urea concentration. The optimal urea concentration for the solubilisation of particulate D,D-carboxypeptidase with the retention of enzymatic activity, in conjuction with Genapol X-100 is shown in Fig. 2. As observed in the absence of detergent, the degree of solubilisation of both enzyme activity and protein increased with urea concentration, although a significant degree of irreversible inactivation of the enzyme occurred after treatment with concentrations greater than 4 M. The optimal urea concentration for the maximum yield of enzyme in an active form was approximately 3.25 M.

d. Optimal detergent concentration. The solubilisation of particulate enzyme by various concentrations of Genapol X-100 was investigated (Fig. 3). Enzyme solubilisation was found to increase substantially between 0.1 and 0.8%, final concentration. Once this latter value had been exceeded, the presence of detergent up to a final concentration of at least 10% did not increase the degree of solubilisation further; neither, however, did it reduce the yield of soluble enzyme. The solubilisation of membrane protein demonstrated similar properties.

The extraction of firmly-associated membrane protein is to some extent dependent upon the ratio of detergent to membrane lipid [20]; thus, greater solubilisation occurs at higher ratios. In these investigations the membrane lipid concentration after the addition of detergent was about 2—4 mg/ml, hence in order to achieve the five- to ten-fold excess of detergent which has been frequently employed [20], Genapol X-100 was added routinely to a final concentration of 2% (20 mg/ml).

e. Solubilisation as a function of pH. The effect of pH on the solubilisation of the D,D-carboxypeptidase and membrane protein is shown in Fig. 4. It is apparent that the degree of solubilisation depends to a considerable extent on the pH employed for extraction; the higher the pH, the greater the solubilisation of both protein and enzymatic activity that is obtained. However, extrac-

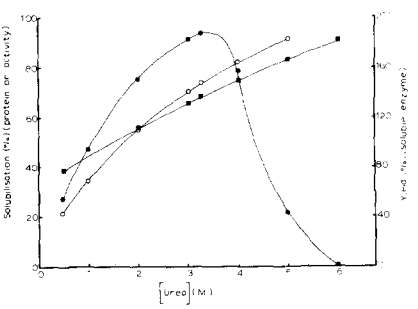
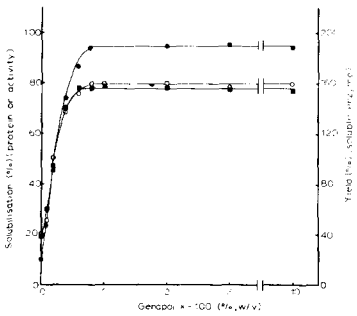


Fig. 2. Effect of urea concentration in the presence of Genapol X-100 on the solubilisation of D,D-carboxypeptidase. Protoplast membranes (3.5 ml; 6.3 mg protein/ml) were treated with 9 M urea to attain the appropriate concentration (37°C, 15 min), and 2% (final concentration, w/v) Genapol X-100 (4°C, 15 min). Percent solubilisation of protein (■) and enzymatic activity (○), and percent yield of active soluble enzyme (●) were determined as described in the legend to Table II.



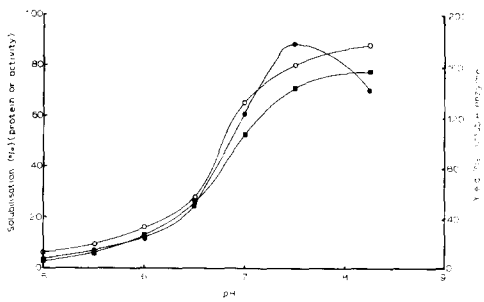


Fig. 3. Effect of Genapol X-100 concentration, in the presence of urea, on the solubilisation of D,D-carboxypeptidase. Protoplast membranes (7.4 mg protein/ml) were treated with 3.25 M (final concentration) urea and Genapol X-100 at the required concentration. Percent solubilisation of protein (■) and enzyme activity (○), and the percentage yield of active soluble enzyme (●) were determined as described previously.

Fig. 4. Effect of pH on the solubilisation of D,D-carboxypeptidase. Protoplast membranes (4.4 mg protein/ml) in 0.05 M buffer at the appropriate pH were treated with 3.25 M urea and 2% Genapol X-100 (final concentration) in 0.05 M buffer at the equivalent pH. Treated controls were retained and the suspensions centrifuged at 225 000 × *g* for 120 min. The soluble fractions and treated controls were dialysed against 0.05 M sodium acetate buffer (pH 4.9) prior to the estimation of D,D-carboxypeptidase activity. Solubilisation was performed in sodium cacodylate-HCl (pH 5.0—7.0) and in Tris-HCl (pH 7.5) and (pH 8.3). Percent solubilisation of protein (■) and enzyme activity (○) and percent yield of active soluble enzyme (●) were determined.

tion at pH 8.3 does cause some inactivation of the enzyme, as the yield of soluble enzyme at this pH was less than that obtained at pH 7.5; thus, more active enzyme was released at the lower pH.

f. Optimal extraction conditions. Experiments to obtain optimal extraction conditions were repeated at least three times; although the actual degrees of solubilisation were found to vary between preparations, the relative degrees of solubilisation by each procedure were similar. Thus the following conditions were adopted:

Membrane preparations (5—12 mg protein/ml) in 0.05 M Tris-HCl, pH 7.5, were treated with 9 M urea to achieve a final concentration of 3.25 M and were stirred at 37°C for 15 min. 8% Genapol X-100 in 0.05 M Tris-HCl, pH 7.5, was added to a final concentration of 2%, and the mixture was stirred at 4°C for a further 15 min. The suspension was then centrifuged at 225 000 × *g* for 2 h, and the resulting supernatant after exhaustive dialysis against 0.05 M Tris-HCl, pH 7.5, was termed the soluble enzyme. Under these conditions, the degree of solubilisation of both enzyme and membrane protein was found to vary between 65 and 90%, while the yield of soluble enzyme relative to untreated membranes varied between 160 and 220%.

The effect of solubilisation on the kinetics of the D,D-carboxypeptidase

The activity of the D,D-carboxypeptidase was stimulated considerably by the incorporation of several non-ionic detergents into incubation mixtures, or upon solubilisation of the membrane-bound enzyme. Enhancement of enzymic activity might be due to an alteration in the Michaelis constant (K_m) or the maximum velocity (V), or to changes in both parameters, and hence such

TABLE III

THE EFFECT OF PHOSPHOLIPASE C ON D,D-CARBOXYPEPTIDASE ACTIVITY

The enzyme source (protoplast membranes or dialysed urea-Genapol X-100-solubilised material (74 and 52 μg protein, respectively)) in 0.02 M sodium cacodylate-HCl, pH 7.4, 2 mM $CaCl_2$ was treated with 2 units of *B. cereus* phospholipase C at 37°C for 3 h, in a volume of 19 μl. Sodium acetate buffer (pH 4.9) and UDP-MurNAc-L-Ala-D-Glu-*meso*-Dap-D-[^{14}C]Ala-D-[^{14}C]Ala were added to final concentrations of 0.05 M and 2 mM, respectively, in a volume of 25 μl; samples were then incubated at 37°C for 20 min.

		Activity (mU)
Protoplast membranes	+ phospholipase C	110.6
	— phospholipase C	54.8
Solubilised enzyme	+ phospholipase C	88.4
	— phospholipase C	85.2

possibilities were investigated by Lineweaver-Burk plots. The K_m and V of the membrane-bound D,D-carboxypeptidase for UDP-MurNAc pentapeptide and pentapeptide, respectively, were 4.6 mM and 14.8 mU/mg protein, and 9.6 mM and 11.3 mU/mg. Treatment of membrane preparations with Genapol X-100 and urea reduced the K_m values to 2.4 and 5.5 mM and increased V to 19.2 and 18.2 mU/mg protein. Similar values were obtained for the solubilised enzyme, which is again indicative that the solubilisation process does not accomplish the specific solubilisation of the D,D-carboxypeptidase relative to total membrane protein.

The effect of *B. cereus* phospholipase C on membrane-bound D,D-carboxypeptidase and urea-Genapol X-100-solubilised enzyme (after dialysis) is shown in Table III. Pretreatment of protoplast membranes with phospholipase C produced an approximately two-fold enhancement of D,D-carboxypeptidase activity; however, no effect was observed on the urea-detergent-solubilised enzyme. The enhanced activity of the membrane-bound enzyme could not be reversed by dialysis and could thus not be attributed to phospholipid degradation products [21].

The effects of phospholipase C stimulation on the kinetics of the enzyme for UDP-MurNAc pentapeptide were investigated. In an analogous manner to urea-Genapol X-100 solubilisation, phospholipase C pretreatment was found to reduce the K_m of the membrane-bound enzyme to 2.5 mM and to increase V to 20 mU/mg protein.

Discussion

The particulate D,D-carboxypeptidase of *B. coagulans* was not released by osmotic shock or sonication and thus confirmed previous results [1] which suggested that the enzyme was firmly associated with the lipoprotein complex of the cytoplasmic membrane.

Urea was not effective in removing much of the D,D-carboxypeptidase from particulate fractions in an active form. Similarly, although the most effective detergents were the non-ionic polyoxyethylated alcohols, Triton X-100, Nonidet P40 (polyoxyethylated octylphenol, where $\bar{n}$ is 10 and 9, respec-

tively), Genapol X-80 and Genapol X-100 (polyoxyethylated tridecanol, where $\tilde{n}$ = 8 and 10), the degree of solubilisation was consistently less than 30%. However, enzyme activity was enhanced greatly in their presence. This situation compared unfavourably with that in other microorganisms in which the great majority of the D,D-carboxypeptidase may be solubilised by detergents [3,4,6, 10—16]. In several instances, solubilisation is also accompanied by an enhancement of enzyme activity [10,12,15,16,22], although this does not always occur [4,6,14].

A considerable correlation exists between the hydrophilic-lipophilic balance (HLB) [23] value of non-ionic detergents and their ability to solubilise biological membranes and membrane-bound enzymes [24—27], including the D,D-carboxypeptidases of *B. subtilis* [22] and *B. stearothermophilis* [12] and the degree of activation of these enzymes obtained upon solubilisation. This is effectively accomplished only by surfactants with HLB values between 12.5 and 14.5. Results indicate that the solubilisation and enhancement of the D,D-carboxypeptidase of *B. coagulans* may also follow this trend. Thus the most effective non-ionic detergents possessed HLB values between 12.6 and 13.8. The structure of Genapol ZDM-090 was not known and hence the HLB value of this surfactant could not be calculated, but it would appear likely to be within this range. However, the chemical structure of the hydrophilic and hydrophobic groups as well as the HLB value has also been shown to be important [20].

Although neither detergents nor urea were able to effect much solubilisation of the membrane-bound enzyme, a combination of both procedures potentiated the degree of solubilisation to such an extent that 65—90% of the available enzyme could be released into the supernatant fraction under optimal conditions. Alteration of the chaotropic agent produced an increased extraction of membrane protein, according to the series $SCN^- > ClO_4^- >$ guanidine-HCl > urea. Nevertheless, the most effective extracting agents also proved to be the most effective inactivators of enzymatic activity, a fact which has been consistently observed [28—30]. The solubilisation of membrane-bound enzymes is facilitated by alkaline pH [11,31,32]; but extensive enzyme solubilisation frequently occurs at pH values which result in their inactivation [31,32]. Thus, in *B. coagulans*, maximal release was obtained at pH 7.5, but beyond this value, although further extraction of membrane protein occurred, the yield of solubilised enzymatic activity was reduced.

The failure of urea or surfactants to solubilise effectively the membrane-bound D,D-carboxypeptidase would suggest either that the enzyme is firmly associated with the lipoprotein matrix of the cytoplasmic membrane, or that the membrane possesses a considerable resistance to disaggregation. The fact that membrane protein, in addition to enzyme activity, was not solubilised by these procedures would support the latter hypothesis. Detergent dissociation of membranes occurs predominantly by the overcoming of hydrophobic interactions; on the other hand, urea affects ionic interactions. It is therefore apparent that the concomitant disruption of both types of interaction is required to destroy the structural integrity of the membrane.

Many membrane-bound enzymes including as described above, several D,D-carboxypeptidases, have been observed to be enhanced by treatment of

particulate fractions with non-ionic detergents [33—35]. In some instances, enhancement has been ascribed merely to the limited permeability of substrate to enzymes located on internal surfaces of membrane vesicles [32]; destruction of the permeability barrier by the detergent will thus apparently activate the enzyme. Sonication of particulate enzyme from *B. coagulans* prior to the assay of D,D-carboxypeptidase activity did not affect the enhancement observed with certain non-ionic detergents and hence activation did not appear to be due to increased substrate permeability. The enhancement of activity and solubilisation of the membrane-bound D,D-carboxypeptidase by such surface active agents was accompanied by an alteration in both the K_m and V of the enzyme, suggesting that a conformational change occurs in the protein after detergent treatment. Surfactant stimulation in *B. stearothermophilus* was also accompanied by an altered enzyme conformation [36]. Changes in enzyme conformation may be induced by direct binding of detergents to the protein [22]; however, similar enzyme activation also occurred on treatment of particulate fractions with phospholipase C. It is thus likely that detergents and phospholipase C remove some constraint present in intact membranes, thereby permitting the D,D-carboxypeptidase to assume a more favourable conformation for enzymic activity. Similar conclusions have been derived to account for enzyme activation by detergents, phospholipases and chaotropic agents in other membrane systems [30,34,35,37,38].

Acknowledgements

The work was carried out during the tenure of a Medical Research Council Research Studentship (H.A.I.M.).

References

1 McArthur, H.A.I. and Reynolds, P.E. (1979) J. Gen. Microbiol. 111, 327—335
2 Blumberg, P.M. and Stroninger, J.L. (1974) Bacteriol. Rev. 38, 291—335
3 Linder, R. and Salton, M.R.J. (1975) Eur. J. Biochem. 55, 291—297
4 Martin, H.H., Maskos, C. and Burger, R. (1975) Eur. J. Biochem. 55, 465—473
5 Davis, R.H. and Salton, M.R.J. (1975) Infect. Immun. 12, 1065—1069
6 Tamura, T., Imae, Y. and Strominger, J.L. (1976) J. Biol. Chem. 251, 414—423
7 Ghuysen, J.M., Léyh-Bouille, M., Frère, J.M., Dusart, J. and Marquet, A. (1974) Ann. N.Y. Acad. Sci. 235, 236—266
8 Gorecki, M., Bar-Eli, A., Burstein, Y., Patchornik, A. and Chain, E.B. (1975) Biochem. J. 147, 131—137
9 Izaki, K. and Strominger, J.L. (1968) J. Biol. Chem. 243, 3193—3201
10 Barnett, H.J. (1973) Biochim. Biophys. Acta 304, 332—352
11 Umbreit, J.N. and Strominger, J.L. (1973) J. Biol. Chem. 245, 6759—6766
12 Yocum, R.R., Blumberg, P.M. and Strominger, J.L. (1974) J. Biol. Chem. 249, 4863—4871
13 Blumberg, P.M. and Strominger, J.L. (1972) Proc. Natl. Acad. Sci. U.S. 69, 3751—3755
14 Pollock, J.J., Nguyen-Distèche, M., Ghuysen, J.M., Coyette, J., Linder, R., Salton, M.R.J., Kim, K.S., Perkins, H.R. and Reynolds, P.E. (1974) Eur. J. Biochem. 41, 439—446
15 Shepherd, S.T., Chase, H.A. and Reynolds, P.E. (1977) Eur. J. Biochem. 78, 521—532
16 Dusart, J., Marquet, A., Ghuysen, J.M. and Perkins, H.R. (1975) Eur. J. Biochem. 56, 57—65
17 Hatefi, Y. and Hanstein, W.G. (1969) Proc. Natl. Acad. Sci. U.S. 62, 1129—1136
18 Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 195, 265—275
19 Warburg, O. and Christian, W. (1941) Biochem. Z. 310, 384—421
20 Helenius, A. and Simons, K. (1975) Biochim. Biophys. Acta 415, 29—79
21 Mookerjea, S. and Yung, J.W.M. (1974) Biochem. Biophys. Res. Commun. 57, 815—822
22 Umbreit, J.N. and Strominger, J.L. (1973) Proc. Natl. Acad. Sci. U.S. 70, 2997—3001

23 Griffin, W.C. (1949) J. Soc. Cosmet. Chem. 1, 311—326
24 Roodyn, D.B. (1962) Biochem. J. 177—189
25 Swanson, P.D., Bradord, H.F. and McIllwain, H. (1964) Biochem. J. 92, 235—247
26 Stromberg, K. (1971) J. Virol. 9, 684—697
27 Hengstenberg, W. (1970) FEBS Lett. 8, 277—280
28 Penefsky, H.S. and Tzagalof, A. (1971) Methods Enzymol. 22, 204—219
29 Chavin, S.I. (1971) FEBS Lett. 14, 269—282
30 Vaino, H. (1973) Biochim. Biophys. Acta 307, 152—161
31 King, T.E. (1963) J. Biol. Chem. 258, 4037—4051
32 Eisenberg, R.C., Yu, L. and Wolin, M.J. (1970) J. Bacteriol. 102, 161—171
33 Ne'eman, Z., Kahane, I. and Razin, S. (1971) Biochim. Biophys. Acta 249, 169—176
34 Srinivasan, R., Korczmar, A. and Bernsohn, J. (1972) Biochim. Biophys. Acta 284, 349—354
35 Chan, P.C. (1967) Biochim. Biophys. Acta 135, 53—60
36 Barnett, H.J. (1973) Ph.D. Thesis, University of Cambridge
37 Marniemi, J. (1974) Chem. Biol. Interactions 9, 135—143
38 Vaino, H. and Hanninen, O. (1974) Acta Pharmacol. Toxicol. 35, 65—75
39 Kagawa, Y. (1972) Biochim. Biophys. Acta 265, 297—338