THE ISOELECTRIC POINT OF PHOSPHOLIPASE C FROM *BACILLUS CEREUS*

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1. Introduction

The exact value of the isoelectric point of phospholipase C (phosphatidylcholine cholinephosphohydrolase, EC 3.1.4.3) from *Bacillus cereus* is a matter of some dispute. Based on observations of the enzyme's behaviour on ion exchange columns, Zwaal et al. [1] concluded that the isoelectric point was between 7.2 and 8.5. However, using isoelectric focusing Otnaess et al. [2] demonstrated a pI value of 6.5. More recently, Ikezawa et al. [3] found two forms of this enzyme in isoelectric focusing with pI values of 6.8 and 7.5. Whilst carrying out isoelectric focusing studies on phospholipase C *(B. cereus)* we observed that even recrystallized enzyme appeared heterogeneous and have investigated this phenomenon further and now present pI values for 3 different forms of this enzyme.

2. Materials and methods

2.1. *Preparation of enzyme samples*

Phospholipase C was isolated from the culture supernatant of *B. cereus* as described previously [4,5]. Purified enzyme was crystallized from ammonium sulphate solution [5]. The crystals were collected and washed three times by resuspension in water and then dissolved and crystallized again from ammonium sulphate solution. The crystals were again washed and finally dissolved in 0.15 M NaCl/3 mM sodium 5,5′-diethylbarbiturate, pH 7.3/0.1 mM $ZnCl_2$. Enzyme samples appeared homogeneous in disc gel electrophoresis under denaturing [7] or non-denaturing [8] conditions. Apoenzyme was prepared by dialysing native enzyme against 0.1 M sodium acetate, pH 6.0/2 mM EDTA for 5 h at room temperature [9].

2.2. *Isoelectric focusing*

Polyacrylamide gels were prepared essentially as described in [10] using a 83 × 65 × 1.5 mm mould. The gels contained 4.9% (w/v) total acrylamide (of which 3% (w/w) was bisacrylamide), 9.9% (w/v) glycerol and either 1.95% (w/v) Ampholine pH 6–8 (LKB) or 1.80% Ampholine pH 3.5–10 plus 0.15% Ampholine pH 5–7 (LKB). Ammonium persulphate (0.024%, w/v) was used as polymerization catalyst. The samples (100–150 μl of 2 mg protein/ml in 1% glycine, 1% glycine/20 μM EDTA or 1% glycine/10 μM $ZnCl_2$) were applied to troughs (10 × 5 × 2.5 mm) in a perspex frame mounted on the gel. Isoelectric focusing was carried out in an LKB Multiphor 2117 apparatus at 10°C and a constant power of 1.5–2.0 W/gel. The electrofocusing time was 2.5 h for the wide pH range and 4 h for the narrow pH range.

After electrofocusing, the pH along the gel was measured at 0.5 cm intervals using an LKB 2117–111 surface pH electrode and a graph was constructed. The gels were fixed and stained with Coomassie Brilliant Blue R 250 as described [10]. When recovery of protein from the gel was desired, a strip along the gel was cut out and briefly fixed. The positions of the most intense bands were detected by dark field illumination and the desired bands were then excised from the non-fixed part of the gel. Corresponding control pieces were cut out 0.5–1 cm from the anode wick. For re-focusing the gel piece containing the isolated band was mounted directly on a new gel near the cathode.

pI values for the different bands were read off from their position on the gel using the above-mentioned position-pH graph. Zinc was determined by atomic absorption spectrometry [11]. Protein was determined by the method of Schaffner and Weissman [12] with the modification that no sodium do-

decylsulphate-Tris buffer was added to the samples. This modification was found necessary to avoid interference by the ampholytes from the isoelectric focusing gel. Bovine serum albumin was used as standard.

3. Results and discussion

When twice crystallized phospholipase C was subjected to isoelectric focusing, one extremely strong band of pI about 7 together with approximately ten other much fainter bands corresponding mainly to components of lower pI values were detected (fig.1a). The strongest of the minor bands, with a stained intensity according to photodensitometric analysis of <5% of that of the major band, had a pI value of about 6. The general pattern of 1 very dominant band of pI ~7 together with about 10 minor bands was found with several batches of crystallized enzyme. The relative intensities of the various minor bands, however, varied from run to run even when the same batch of enzyme was used. Since the enzyme samples used were homogeneous in two different gel electrophoretic systems and had been twice crystallized, this multiplicity of bands is somewhat surprising. It might indicate a heterogeneous sample or alternatively denatured forms of the enzyme produced during the isoelectric focusing. Consequently, a sample of enzyme was subjected to isoelectric focusing and afterwards an area of gel corresponding to the major band was excised and refocused.

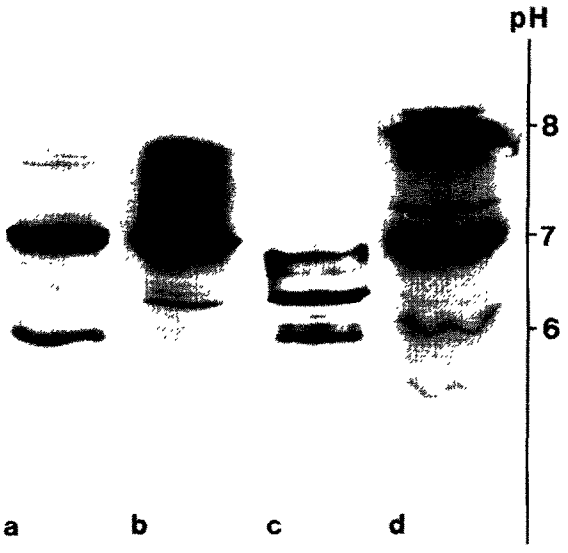

Fig.1. Thin layer isoelectric focusing of recrystallized phospholipase C using pH 3.5–10 ampholytes: (a) native enzyme; (b) the pH 7 band refocused; (c) zinc-free apoenzyme; (d) native enzyme with Zn^{2+} present in the gel.

On refocusing, this band yielded one very intense band of pI ~7 together with apparently the previous ten or so other faint bands of lower pI values (fig.1b). In addition, several other generally diffuse bands of pI values >7 were discernable. It therefore seems that most, if not all the minor bands observed in isoelectric focusing of this enzyme represent denatured/modified conformations formed during the experiment rather than contaminating proteins in the original enzyme sample. In the rerun sample, the pI 6 band was, however, very much fainter than in the original run (cf. fig.1a and 1b). In addition to producing the present (phosphatidylcholine-hydrolyzing) phospholipase C, *B. cereus* also synthesizes a sphingomyelinase C and a phosphatidylinositol-hydrolyzing phospholipase C [3,13]. The pI values for these enzymes are 5.6 ± 0.1 and 5.4 ± 0.1 respectively [3]. The present samples of crystalline phosphatidylcholine-hydrolyzing phospholipase C produced some extremely faint bands of pI value around 5.5. However, on rerunning the main band, some very faint bands in the same pI region were apparent. It is therefore unlikely that the two other phospholipase C-type enzymes were present as significant contaminants.

Phospholipase C contains two Zn atoms [11] which contribute greatly to the enzymes' high conformational stability [14,15]. When the zinc-free apoenzyme was run in isoelectric focusing the main band now had a pI value of about 6.3 and again approx. 10 minor bands were found (fig.1c). The two strongest of the minor bands had pI values of ~7.0 (presumably native enzyme) and 6.0. It is interesting that in fig.1b with the rerun pI 7 band, a band of pI 6.3 is the strongest minor band. This would suggest that the enzyme has a tendency to lose zinc during the run.

When a low level of Zn^{2+} (10 μM) is included in the gel, the pI value of the major band from phospholipase C becomes 7.9 (table 1), but a strong minor band of pI 7 is still present together with many fainter bands (fig.1d). The pI 7.9 band was occasionally clearly visible even when Zn^{2+} was not included in the gel.

Table 1
Properties of three forms of phospholipase C

Form	pI	Zinc content (mol/mol of enzyme)
Apoenzyme	6.29 ± 0.05	0
Native enzyme	6.88 ± 0.05	2.04
Zn^{2+}-enzyme	7.9 ± 0.1	8.7

Isoelectric focusing was carried out with pH 6–8 ampholytes (see section 2) on native and apophospholipase C. In the case of the Zn^{2+}-enzyme, 10 μM $ZnCl_2$ was present in the gel and pH 3.5–10 ampholytes were used. For the zinc content of the enzyme forms, an area of gel corresponding to the major band was excised, broken up and extracted in 5 ml 0.5 M NaCl for 2 h at 22°C prior to zinc and protein determination. In each case an equivalent area of gel away from the major peak was excised, extracted and used as control. The Zn^{2+}-enzyme band was obtained from a gel to which no free Zn^{2+} had been added, but where this pI 7.9 band was unusually strong

More accurate values for the isoelectric points of the major bands produced by the apoenzyme and the native enzyme were obtained using pH 6.0–8.0 ampholytes in the gel and are given in table 1. The zinc content of these bands confirms the identity of the apoenzyme and the native enzyme. The zinc content of the pI 7.9 band was 8–9 mol Zn/mol of enzyme, suggesting that in addition to having two very tight binding sites for zinc, the enzyme has another 6–7 weaker binding sites for the metal (table 1). Preliminary equilibrium dialysis studies also suggest some 6–8 weaker binding sites for zinc on the enzyme (C. Little, unpublished work).

Thus, native phospholipase C, which we may regard as the enzyme form containing only the two most tightly bound zinc atoms has a pI value of 6.88 ± 0.05. This corresponds well with the pI value of 6.8 ± 0.1 found by Ikezawa et al. [3] for the major form of this enzyme found in isoelectric focusing. Using pure enzyme we find no significant amount of an enzyme form corresponding to the pI 7.5 ± 0.1 phospholipase C activity reported by Ikezawa et al. [3] using a very crude enzyme preparation. Zwaal et al. [1] concluded from the behaviour of the enzyme on ion exchange columns that the pI value was between 7.2 and 8.5. Although these workers had no free Zn^{2+} present, 5 mM Ca^{2+} was present throughout the chromatographic studies. It would therefore seem possible that Ca^{2+} binds to the low affinity binding sites for Zn^{2+} to produce an enzyme form with an elevated isoelectric point equivalent to the pI 7.9 form observed in the presence of free Zn^{2+}.

The discrepancy between the present pI value for native enzyme and the value of 6.5 obtained by Otnaess et al. [2] is more difficult to explain. It is possible that the particular mutant of *B. cereus* used in that work [2] may synthesize a slightly different form of phospholipase C.

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