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A NOVEL METHOD FOR THE PURIFICATION OF PORCINE PHOSPHOLIPASE A_2 EXPRESSED IN E.COLI

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Porcine phospholipaseA_2 expressed in E.coli as a fusion protein was isolated, renatured and specifically cleaved by trypsin as described in (1). Active phospholipaseA2, was purified to homogeneity on a column of PBE-94 over a pH region 7.4-4.5. Using this method, several phospholipase A_2 mutant enzymes have now been purified in a single step and all behaved identically during chromatofocusing. The method will therefore be extremely useful not only for those interested in understanding the structure-function relationships of phospholipaseA_2 but also for preparing the enzyme in large quantities for industrial and pharmaceutical purposes.

PhospholipasesA_2 (EC 3.1.1.4) are a family of lipolytic enzymes, which in the presence of calcium, specifically catalyse the hydrolysis of the 2-acyl ester bond of 3-sn-phosphoglycerides, including all naturally occuring phospholipids (2). It plays a key role in a number of physiologically important cellular processes namely inflamation, blood platelet aggregation and acute hypersensitivity (3,4). Furthermore, phospholipaseA_2 has tremendous commercial potential in the preparation of food emulsifiers as well as in upgrading low quality fats and oils(5). Although these interests resulted in considerable research activity in this area in recent years, knowledge on structure-function relationships of phospholipaseA_2 is limited.

Our objectives include the modification of the stability and specificity of phospholipaseA_2 in order to elucidate the structure-function relationships of this enzyme using a more rational approach than hitherto. For this reason, we have used an E.coli expression system, where phospholipaseA_2 cDNA has been cloned downstream from a portion of β-

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galactosiadse gene and expressed as a fusion protein under the control of the lambda pR promotor. Desired modifications, based on computer modelling and electrostatic calculations were then introduced by site-directed mutagenesis, changing either one or two amino acids. The effect of such changes on the properties of phospholipaseA_2 were studied biochemically. Such studies may also provide some new information on the mechanism of action of phospholipaseA_2.

A limiting feature in these studies is the availability of a simple and rapid method which can reduce the interaction of phospholipaseA_2 and trypsin during purification. Because the trypsin added during activation step could further degrade an intact and fully active phospholipaseA_2, it is important to keep their interaction minimal during purification. The method described in the present paper achieves such a goal and provides a homogeneous protein in a single step.

Materials and Methods

Materials: The phospholipaseA_2 expression system, comprising E.coli strain AB1157, pCI857 (a source of the temperature variant of the lambda repressor CI) and pOK13.2 (containing the phospholipaseA_2 cDNA, cloned downstream from a portion of the β-galactosidase gene and expressed as a fusion protein under the control of the lambda pR promoter) was a gift of Dr.H.M.Verheij, Department of Biochemistry, University of Utrecht, Netherlands. PhospholipaseA_2 mutants were produced by site-directed mutagenesis as described(6). Polybuffer ion-exchanger-94 and polybuffer-74 were from Pharmacia, Uppsala,Sweden while acrylagel and bisacrylagel were from National diagnostics, Aylesbury, Berks, U.K. Remaining chemicals were either from BDH Chemicals, Poole, Dorset, U.K. or from Sigma Chemicals, Poole, Dorset, U.K.

Production of phospholipaseA_2-fusion protein as inclusion bodies: E.coli strain AB1157, carrying both pCI857 and either a negative or mutated form of the pOK13.2 expression plasmid was cultured in a fermentor using Luria broth medium (10L), without Mg^{++} at 30°C until the O.D at 595nm reached approximately 0.8. Production of fusion protein was then induced by temperature shock at 41°C for about 3hrs prior to harvesting. Cells were concentrated using a Sartorius cell (Sartocon mini) fitted with a cellulose acetate membrane (pore size-2μ) followed by centrifugation at 6000xg for 10 min.

Isolation of inclusion bodies: A 20% (w/v) cell suspension, prepared in 50 mM Tris-HCl buffer pH 8.0 containing 50 mM NaCl, 1mM EDTA and 0.5mM PMSF was made up to 0.4% (w/v, final conc.) with respect to Triton X-100 and sodium deoxycholate and stirred for 20 min. at 4°C prior to french pressing at 12000 psi. The process was repeated after increasing the final concentration of both Triton X-100 and sodium deoxycholate to 0.8% (w/v). The resultant sample on centrifugation (6000xg for 30min.), separated into three fractions, namely supernatant, fluffy pellet and hard pellet. The hard

pellet, rich in inclusion bodies, was separated and washed with above buffer containing 1% Triton X-100. Subsequent washings were carried out using the above buffer without Triton X-100.

Solubilization of inclusion bodies and renaturation: Inclusion bodies were solubilized in 6M guanidine HCl, pH 8.0 and the soluble protein was clarified and sulphonated as described(7). Sulphonated protein was precipitated by 1% acetic acid, washed and solubilized in 25mM borate and 5mM EDTA buffer, pH 8.0 containing 5M urea. Renaturation was effected at room temperature (24h) by diluting the sample using the above buffer (1:5), containing 1 and 2 mM reduced and oxidized glutathione, respectively. Resultant sample was then dialysed against 20 mM Tris-HCl buffer and concentrated (2mg-3mg/ml).

Activation of phospholipaseA_2: The renatured protein was made 15mM with respect to CaCl2 at pH 8.0 and cleaved by trypsin (1/250) with stirring at 4°C. Production of active phospholipaseA_2 was followed by measurement of activity using mixed micellar (egg yolk) substrate. The reaction was terminated after the activity reached maximum (by about 2hrs), by adding soyabean trypsin inhibitor (twice the amount of trypsin), PMSF (1mM, final conc.), followed by adjusting the pH to 5.0 using 10% acetic acid. The resultant sample was dialysed (10mM Na acetate buffer, pH 5.0) and active phospholipaseA_2 present in the supernatant was isolated by centrifugation at 30000xg.

Purification of phospholipaseA_2 by chromatofocusing: Active phospholipase A_2 was concentrated using an Amicon cell fitted with YM-2 membrane, dialysed against 25mM imidazole-HCl buffer,pH 7.4 containing 0.5mM PMSF and applied on to a column (48X1.6cm) of PBE-94, previously equilibrated with the above buffer. The column was then eluted (30ml/h)with 800 ml of polybuffer-74 (diluted (1:8) and adjusted the pH to 4.5 with 1N HCl). Fractions (5ml) collected were analysed for protein, enzyme activity and pH. Polybuffer associated with enzyme sample was readily removed by dialysis.

Activity measurement of phospholipase A_2 using egg yolk lecithin:This was measured titrimetrically as described (8), using egg yolk lecithin as substrate, in the presence of Ca^{++}(10mM, final conc.), deoxycholate (2.6 mM, final conc.) at 37°C, pH 8.0.

SDS-polyacrylamide gel electrophoresis: Electrophoresis was as described(9), using a gradient (10-15%) gel. The gel was stained using 0.25% coomassie blue prepared in propanol:acetic acid:water (3:1:6) and destained in propanol:acetic acid:water(3:1:6).

Results and Discussion

Isolation and renaturation of phospholipaseA_2-fusion protein:

Porcine phospholipaseA_2, expressed in E.coli as a fusion protein, was prepared as described in the methods section. Each fermentor run yielded about 50 g cell paste per 10 litre medium. SDS-PAGE analysis of culture samples withdrawn at different time intervals after induction showed an initial increase in fusion protein levels and reached a maximum by about

3hrs. Visual observation of SDS-PAGE suggested that the fusion protein constituted about 5-10% of the total cell protein (data not shown). Attempts to improve the yield by changing either fermentation conditions (agitation, aeration) or medium composition (glucose and Mg^{++} concentrations) did not show any further significant increase in the levels of fusion protein. Although an obvious reason for such low yield is not known, it is often reported that when the bacterial gene constitutes a large proportion of the fusion protein, the amount of eukaryotic product is always small(10) and this may well be the case with phospholipaseA_2.

Many eukaryotic proteins, including phospholipaseA_2, when expressed in prokaryotes as a fusion protein generally accumulate in an insoluble form as inclusion bodies(10). One of the advantages of inclusion bodies is that they can be readily isolated as a hard pellet by low speed centrifugation of a cell lysate. SDS-PAGE analysis of the hard pellet revealed that it contained >50% of total protein as fusion protein. However, the disadvantages of this form of production include a requirement for additional steps during purification such as solubilzation and renaturation. PhospholipaseA_2 expressed in E.coli as an insoluble protein has been successfully solubilized and renatured as described in methods. The yield of renatured protein at this stage was generally in excess of 3-5g per 100 g of cell paste.

Activation and purification of phospholipaseA_2:

Renatured phospholipaseA_2-fusion protein was inactive when assayed using egg yolk lecithin as substrate. However, the activity appeared soon after the addition of trypsin and reached a maximum by about 2hours after which the reaction was terminated as described in Methods. A large amount of protein was precipitated during acidification and dialysis against 10 mM sodium acetate buffer, pH 5.0, but more than 95% of the total phospholipaseA2 activity was present in the supernatant. Further analysis of this supernatant by SDS-PAGE revealed that the phospholipaseA2 preparation is contaminated mainly by trypsin, trypsin inhibitor and a few other low molecular weight proteins.

A search of the literature revealed that phospholipaseA_2, trypsin and trypsin inhibitor differed from one another significantly with respect to their pI values, being 5.6, 9.3 and 4.5, respectively (11-13). Although these results suggest that phospholipaseA_2 could be easily separated from trypsin and trypsin inhibitor, the possibility that the enzyme could still be contaminated by other E.coli protein can not be ruled out unequivocally. This was tested by isolectric focusing of supernatant sample in polyacrylamide gels over a pI region 3-10. It was observed that phospholipaseA2 was well separated from all other contaminating proteins

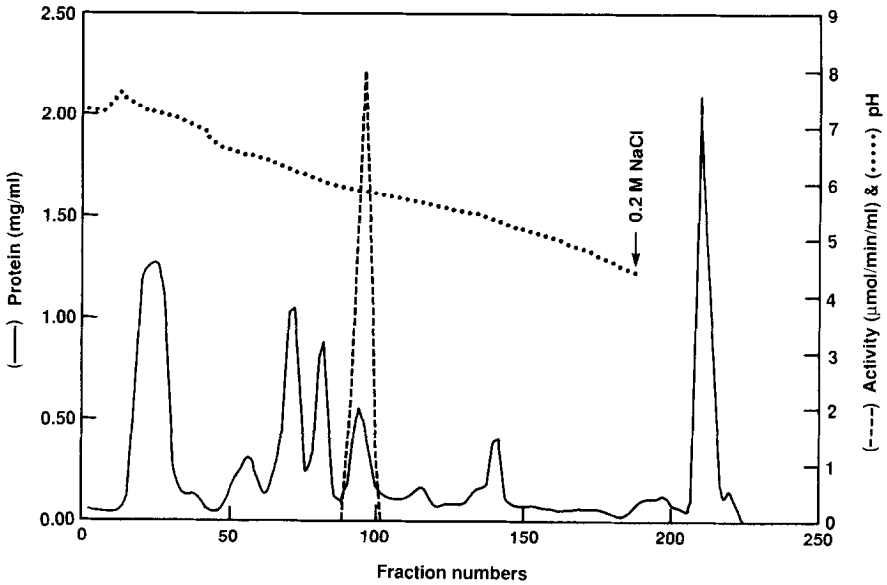

Fig.1. Purification of phospholipaseA_2 expressed in E.coli by chromatofocusing. A 260mg protein sample containing active phospholipaseA_2 (double mutant) was concentrated, dialysed and chromatofocused as described in the methods sections. Fractions (5ml) collected were analysed for protein (-), activity (--) and pH (••).

over the pI region 3-10.(data not shown). These results further confirmed that phospholipaseA_2 could be easily purified by chromatofocusing.

Fig.1 shows a clean separation of phospholipaseA_2 (double mutant) from trypsin, trypsin inhibitor and a few other contaminating proteins during chromatofocusing over the pH region 7.4-4.5. PH measurement of individual fractions revealed that phospholipaseA_2 from the double mutant eluted in the region 5.6-5.4. Similar elution patterns were observed for wild type and other mutant phospholipaseA_2 preparations during chromatofocusing under identical conditions. Analysis of fractions corresponding to different peaks by SDS-PAGE revealed that trypsin and trypsin inhibitor were associated with first and last protein peaks, respectively while the phospholipaseA_2 protein peak (peak no.5) contained only one protein species of molecular weight approximately 14K. Furthermore, a comparison of purified phospholipaseA_2 from single (N89D) and double (N89D/E92K) mutants with native Porcine phospholipaseA2 by SDS-PAGE showed that both preparations were homogeneous with apparently similar molecular weights as native phospholipaseA_2 (Fig.2).

The method described in the present paper, unlike a multistage stage purification procedure reported previously(1,14), provided a homogeneous recombinant phospholipaseA_2 in a single step. Moreover, the chromatofocusing step used in the present study showed no binding of

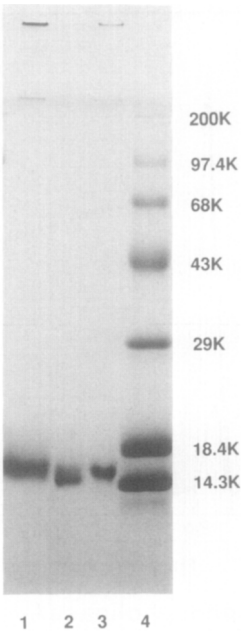

Fig.2. SDS-polyacrylamide gel electrophoresis of phospholipaseA_2. Lane1, native Porcine phospholipaseA_2 (10μg); lane2, phospholipaseA_2-single mutant(N89D, 10μg); lane3, phospholipaseA_2-double mutant(N89D/E92K, 10μg) and lane4, standard marker proteins in order of increasing molecular mass, lysozyme, β-lactoglobulin, carbonic anhydrase,ovalbumin, albumin, phosphorylase B and myosin (H-chain) (15μg).

trypsin to polybuffer ion-exchanger-94 and thus avoiding any slightest possibility of degradation of active phospholipaseA_2 during purification.

In conclusion, we have developed a simple and rapid method for the purification of phospholipaseA_2 to homogeneity in a single step. Furthermore, these results strongly suggest that chromatofocussing could be the method of choice for other protease activated enzyme where the isolectric points are well separated. Thus, the method will clearly be of interest for all those involved either in basic or applied research in the area of phospholipaseA_2 and related enzymes.

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