

# Identification of the bovine brain Ins(1,4,5)P<sub>3</sub> 5-phosphatase after SDS-polyacrylamide gel electrophoresis

Manuela Lemos, Jacques E. Dumont and Christophe Erneux

*Institut de Recherche Interdisciplinaire (IRIBHN), Faculté de Médecine, Université Libre de Bruxelles, Campus Erasme, Route de Lennik 808, 1070 Bruxelles, Belgium*

Received 10 April 1989

Ins(1,4,5)P<sub>3</sub> 5-phosphatase catalyzes the dephosphorylation of Ins(1,4,5)P<sub>3</sub> in the 5-position. In a high speed soluble fraction of bovine brain, there are two soluble 5-phosphatases: type I and type II. The purified Ins(1,4,5)P<sub>3</sub> 5-phosphatase type I exhibits a major silver-stained band of 43 kDa on denaturing (SDS) gels. It is possible to extract the 5-phosphatase activity from a duplicate lane after gel electrophoresis. The 43 kDa region contains the extractable Ins(1,4,5)P<sub>3</sub> 5-phosphatase activity.

Ca<sup>2+</sup>; Inositol 1,4,5-trisphosphate 5-phosphatase; (Bovine brain)

## 1. INTRODUCTION

Ins(1,4,5)P<sub>3</sub>, a second messenger for mobilizing intracellular calcium [1,2], has been shown to be metabolized by two key enzymes: Ins(1,4,5)P<sub>3</sub> 5-phosphatase, which catalyzes the dephosphorylation of Ins(1,4,5)P<sub>3</sub> in the 5-position, and Ins(1,4,5)P<sub>3</sub> 3-kinase, which catalyzes the formation of Ins(1,3,4,5)P<sub>4</sub> [3]. Recent evidence indicates that Ins(1,3,4,5)P<sub>4</sub> shows specific biological effects. A synergism between Ins(1,4,5)P<sub>3</sub> and Ins(1,3,4,5)P<sub>4</sub> has been proposed as a control mechanism of calcium entry from outside the cell or inside the cell of calcium exchange between two calcium pools ([4,5] and discussed in [6]). In bovine brain (and many other tissues, see [7] for review), Ins(1,4,5)P<sub>3</sub> 5-phosphatase is mostly

associated to the particulate fraction. A high speed soluble fraction indicates the presence of two soluble 5-phosphatases: type I and type II [8,9]. Type I soluble enzyme and the particulate Ins(1,4,5)P<sub>3</sub> 5-phosphatase dephosphorylate both Ins(1,4,5)P<sub>3</sub> and Ins(1,3,4,5)P<sub>4</sub> with exactly the same kinetics ( $K_m$  values and  $V_{max}$  ratio for both substrates [9]).

We have previously reported the purification of type I Ins(1,4,5)P<sub>3</sub> 5-phosphatase in bovine brain [9]. In this paper, we identify the protein after SDS-polyacrylamide gel electrophoresis. The 5-phosphatase exhibits a major silver-stained band with an apparent molecular mass of 43 kDa.

## 2. MATERIALS AND METHODS

### 2.1. Materials

Chemicals were obtained as cited previously [9].

### 2.2. Purification of bovine brain Ins(1,4,5)P<sub>3</sub> 5-phosphatase

Ins(1,4,5)P<sub>3</sub> 5-phosphatase (type I) was purified as previously described by DEAE Sephacel, followed by Blue Sepharose, Sephacryl S-200 and phosphocellulose [9]. After phosphocellulose, the enzyme (1 mg) was dialyzed against 20 mM Tris-HCl, pH 7.5, 0.1 mM EGTA, 2 mM MgCl<sub>2</sub>, 10% glycerol, 0.4 mM phenylmethanesulfonyl fluoride and 5  $\mu$ M leupeptin (buffer B),

*Correspondence address:* M. Lemos, Institut de Recherche Interdisciplinaire (IRIBHN), Faculté de Médecine, Université Libre de Bruxelles, Campus Erasme, Route de Lennik 808, 1070-Bruxelles, Belgium

*Abbreviations:* Ins(1,4,5)P<sub>3</sub>, inositol 1,4,5-trisphosphate; Ins(1,3,4,5)P<sub>4</sub>, inositol 1,3,4,5-tetrakisphosphate

diluted 5-fold and applied to a Blue Sepharose column ( $2.8 \times 0.7$  cm) at a flow rate of 30 ml/h. Fractions were of 0.9 ml. The column was washed with 10 ml of buffer B followed by 10 ml of 0.1 M NaCl in buffer B and 10 ml of 10 mM 2,3-bisphosphoglycerate in buffer B. 5-Phosphatase was eluted with both the 0.1 M NaCl salt and the bisphosphoglycerate eluate (80–90% recovery of activity at  $30 \mu\text{M}$   $\text{Ins}(1,4,5)\text{P}_3$  substrate level). The fractions were analyzed by SDS-polyacrylamide gel electrophoresis and proteins were visualized by the silver-stain procedure [10]. The fractions containing the highest specific activity were pooled (3.6 ml) and concentrated by speed vac to 1 ml. Protein concentrations were estimated by the procedure of Bradford [11]. 5-Phosphatase activity was determined as previously described [9]. Specific activities of purified type I  $\text{Ins}(1,4,5)\text{P}_3$  5-phosphatase were 20–40  $\mu\text{mol}/\text{min}$  per mg of protein at  $37^\circ\text{C}$  and  $30 \mu\text{M}$   $\text{Ins}(1,4,5)\text{P}_3$ . We calculated the purification to be approximately 3000-fold as compared to the crude soluble fraction.

### 2.3. SDS-polyacrylamide gel electrophoresis

All steps were performed at  $4^\circ\text{C}$ . Enzyme preparation was made 62 mM Tris-HCl, pH 6.8, 3% SDS, 5% 2-mercaptoethanol, 10% glycerol 'sample buffer' and immediately run (without boiling) on a SDS 10% polyacrylamide mini slabgel [12]. After electrophoresis at 200 V (60 min), the gel was cut into two parts: one part was silver-stained, the other was sliced into 2 mm sections. Each slice was homogenized in 0.5 ml or 1 ml of 84 mM Hepes/NaOH, pH 7.5, 1 mg/ml bovine serum albumin, 12 mM 2-mercaptoethanol and 25% sucrose. After 12 h incubation at  $4^\circ\text{C}$ , a sample of each fraction was assayed for activity in the presence of 1% Triton X-100 for 30–60 min incubation at  $37^\circ\text{C}$ . Recovery of enzyme activity was about 30–60% of the material loaded on the gel in several separate experiments.

## 3. RESULTS AND DISCUSSION

In the following experiments, we aimed to identify the purified  $\text{Ins}(1,4,5)\text{P}_3$  5-phosphatase (spec. act. 20–40  $\mu\text{mol}/\text{min}$  per mg of protein) after SDS-polyacrylamide gel electrophoresis. Preliminary experiments indicated that the anionic detergent SDS strongly inhibited  $\text{Ins}(1,4,5)\text{P}_3$  5-phosphatase activity. At 0.1% SDS, the enzyme was totally inactive. However, this effect could be partially reversed provided the assay was performed in the presence of a nonionic detergent Triton X-100 at 1%. When added to 'sample buffer',  $\text{Ins}(1,4,5)\text{P}_3$  5-phosphatase activity was 20% of its control value provided the material was not boiled (not shown). We concluded from these data that the material subjected to SDS-polyacrylamide gel electrophoresis showed  $\text{Ins}(1,4,5)\text{P}_3$  5-phosphatase activity. Other enzymes, e.g. phosphatidylinositol kinase from bovine brain myelin

or from bovine uteri could be extracted after SDS-polyacrylamide gel electrophoresis [13,14]. After electrophoresis, one lane was sliced into 2-mm sections as described in section 2.  $\text{Ins}(1,4,5)\text{P}_3$  5-phosphatase was associated with a major peak of activity with an apparent molecular mass of 43 kDa. Silver staining from a duplicate lane demonstrated a single major protein with the same  $M_r$  (fig.1). The

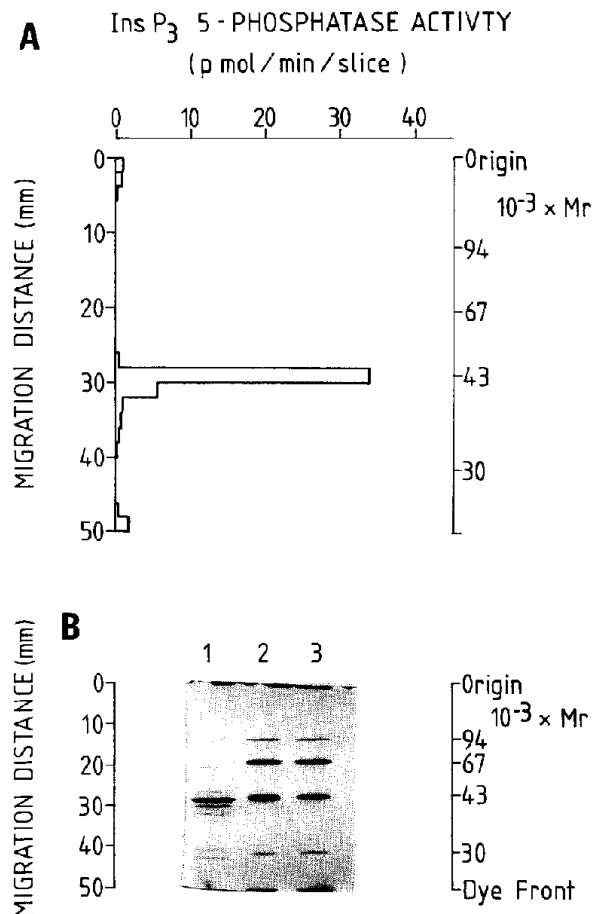


Fig.1. SDS-polyacrylamide gel of purified  $\text{Ins}(1,4,5)\text{P}_3$  5-phosphatase. (A) Localization of  $\text{Ins}(1,4,5)\text{P}_3$  5-phosphatase after SDS-polyacrylamide gel electrophoresis. One lane (50 mm length) was cut into 2 mm sections, extracted and assayed for activity.  $\text{Ins}(1,4,5)\text{P}_3$  concentration was  $1 \mu\text{M}$  and assay mixture contained 1% Triton X-100. (B) The other part of the electrophoresis was silver-stained. Lane 1 contains  $0.4 \mu\text{g}$  of purified  $\text{Ins}(1,4,5)\text{P}_3$  5-phosphatase. Lanes 2 and 3 contain protein standards consisting of  $0.09 \mu\text{g}$  of phosphorylase *b* ( $M_r$  94000),  $0.12 \mu\text{g}$  of bovine serum albumin ( $M_r$  67000),  $0.21 \mu\text{g}$  of ovalbumin ( $M_r$  43000) and  $0.12 \mu\text{g}$  of carbonic anhydrase ( $M_r$  30000).

purified enzyme eluted as a single component from a Sephacryl S-200 column with an apparent molecular mass of  $37 \pm 3$  kDa [9]. Taken together, these results suggest that the bovine brain Ins(1,4,5)P<sub>3</sub> 5-phosphatase type I is a monomer. These results are comparable to the data reported by Connolly and Majerus for the Ins(1,4,5)P<sub>3</sub> 5-phosphatase of human platelets [15]. Their purified enzyme migrated with an apparent molecular mass of 45 kDa [3] on SDS gels, but the enzyme maximal specific activity was 10–20-fold lower as compared to the bovine brain enzyme [15].

In conclusion, to obtain partial amino acid sequence information of the Ins(1,4,5)P<sub>3</sub> 5-phosphatase and specific probes [16], it was essential to identify the protein on SDS gels. The purified Ins(1,4,5)P<sub>3</sub> 5-phosphatase type I of bovine brain exhibits a major silver-stained band of 43 kDa on denaturing gels. The data presented here demonstrate that the 43 kDa region contains the extractable Ins(1,4,5)P<sub>3</sub> 5-phosphatase activity.

*Acknowledgements:* This work was supported by Duphar (Holland) and under contract of the Ministère de la Politique Scientifique (Belgium). We would like to thank Dr R. Lecocq for advice on the silver-stain technique and Dr K. Takazawa and B. Robaye for their helpful criticism. The excellent technical assistance of C. Moreau is also gratefully acknowledged.

## REFERENCES

- [1] Berridge, M.J. and Irvine, R.F. (1984) *Nature* 312, 315–321.
- [2] Irvine, R.F. (1986) *Br. Med. Bull.* 42, 369–374.
- [3] Majerus, P.W., Connolly, T.M., Bansal, V.S., Inhorn, R.C., Ross, T.S. and Lips, D.L. (1988) *J. Biol. Chem.* 263, 3051–3054.
- [4] Irvine, R.F. and Moor, R.M. (1986) *Biochem. J.* 240, 917–920.
- [5] Morris, A.P., Gallacher, D.V., Irvine, R.F. and Petersen, O.H. (1987) *Nature* 330, 653–655.
- [6] Irvine, R.F., Moor, R.M., Pollock, W.K., Smith, P.M. and Wreggett, K.A. (1988) *Phil. Trans. R. Soc. London B320*, 281–298.
- [7] Shears, S.B. (1989) *Cell. Signal.* 1, 125–133.
- [8] Hansen, C.A., Johanson, R.A., Williamson, M.T. and Williamson, J.R. (1987) *J. Biol. Chem.* 262, 17319–17326.
- [9] Erneux, C., Lemos, M., Verjans, B., Vanderhaeghen, P., Delvaux, A. and Dumont, J.E. (1989) *Eur. J. Biochem.*, in press.
- [10] Merrill, C.R. and Goldman, D. (1984) in: *Two Dimensional Gel Electrophoresis of Proteins. Methods and Applications* (Celis, J.E. and Bravo, R. eds) pp.93–109, Academic Press, Inc., Orlando, FL.
- [11] Bradford, M.M. (1976) *Anal. Biochem.* 72, 248–254.
- [12] Laemmli, U.K. (1970) *Nature* 227, 680–685.
- [13] Saltiel, A.R., Fox, J.A., Sherline, P., Sahyoun, N. and Cuatrecasas, P. (1987) *Biochem. J.* 241, 759–763.
- [14] Porter, F.D., Li, Y.-S. and Deuel, T.F. (1988) *J. Biol. Chem.* 263, 8989–8995.
- [15] Connolly, T.M., Bross, T.E. and Majerus, P.W. (1985) *J. Biol. Chem.* 260, 7868–7874.
- [16] Kennedy, T.E., Wagger-Smith, K., Barzilai, A., Kandel, E.R. and Sweatt, J.D. (1988) *Nature* 336, 499.