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Identification of the bovine brain Ins(1,4,5)P_3 5-phosphatase after SDS-polyacrylamide gel electrophoresis

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Ins(1,4,5)P_3 5-phosphatase catalyzes the dephosphorylation of Ins(1,4,5)P_3 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_3 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 form a duplicate lane after gel electrophoresis. The 43 kDa region contains the extractable Ins(1,4,5)P_3 5-phosphatase activity.

Ca^{2+}; Inositol 1,4,5-trisphosphate 5-phosphatase; (Bovine brain)

1. INTRODUCTION

Ins(1,4,5)P_3, a second messenger for mobilizing intracellular calcium [1,2], has been shown to be metabolized by two key enzymes: Ins(1,4,5)P_3 5-phosphatase, which catalyzes the dephosphorylation of Ins(1,4,5)P_3 in the 5-position, and Ins(1,4,5)P_3 3-kinase, which catalyzes the formation of Ins(1,3,4,5)P_4 [3]. Recent evidence indicates that Ins(1,3,4,5)P_4 shows specific biological effects. A synergism between Ins(1,4,5)P_3 and Ins(1,3,4,5)P_4 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_3 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_3 5-phosphatase dephosphorylate both Ins(1,4,5)P_3 and Ins(1,3,4,5)P_4 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_3 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_3 5-phosphatase*

Ins(1,4,5)P_3 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 $MgCl_2$, 10% glycerol, 0.4 mM phenylmethanesulfonyl fluoride and 5 μM leupeptin (buffer B),

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Abbreviations: Ins(1,4,5)P_3, inositol 1,4,5-trisphosphate; Ins(1,3,4,5)P_4, inositol 1,3,4,5-tetrakisphosphate

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diluted 5-fold and applied to a Blue Sepharose column (2.8 × 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 μM Ins(1,4,5)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 Ins(1,4,5)P_3 5-phosphatase were 20–40 μmol/min per mg of protein at 37°C and 30 μM Ins(1,4,5)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°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°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°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 Ins(1,4,5)P_3 5-phosphatase (spec. act. 20–40 μmol/min per mg of protein) after SDS-polyacrylamide gel electrophoresis. Preliminary experiments indicated that the anionic detergent SDS strongly inhibited Ins(1,4,5)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', Ins(1,4,5)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 Ins(1,4,5)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. Ins(1,4,5)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

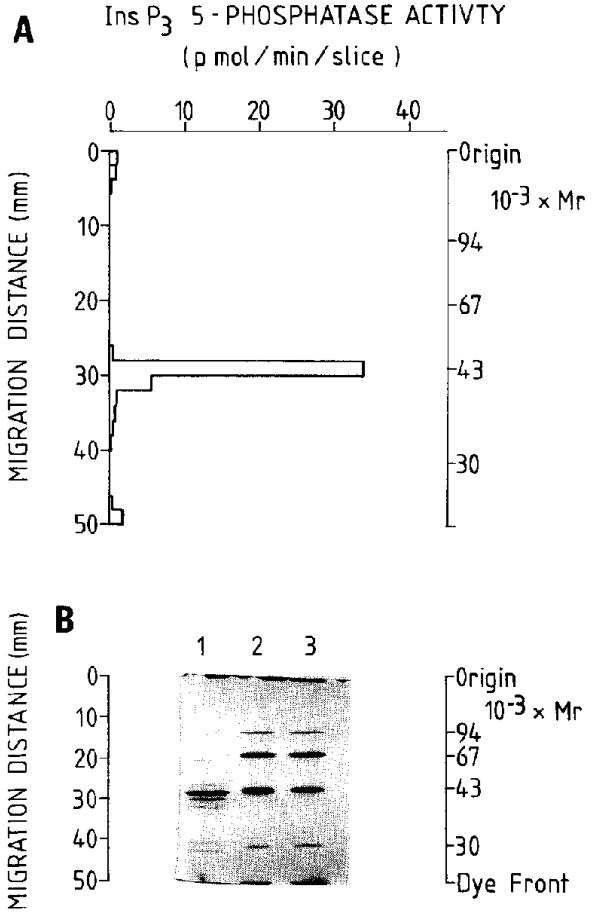

Fig.1. SDS-polyacrylamide gel of purified Ins(1,4,5)P_3 5-phosphatase. (A) Localization of Ins(1,4,5)P_3 5-phosphatase after SDS-polyacrylamide gel electrophoresis. One lane (50 mm length) was cut into 2 mm sections, extracted and assayed for activity. Ins(1,4,5)P_3 concentration was 1 μM and assay mixture contained 1% Triton X-100. (B) The other part of the electrophoresis was silver-stained. Lane 1 contains 0.4 μg of purified Ins(1,4,5)P_3 5-phosphatase. Lanes 2 and 3 contain protein standards consisting of 0.09 μg of phosphorylase *b* (M_r 94000), 0.12 μg of bovine serum albumin (M_r 67000), 0.21 μg of ovalbumin (M_r 43000) and 0.12 μg 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 ± 3 kDa [9]. Taken together, these results suggest that the bovine brain Ins(1,4,5)P_3 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_3 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_3 5-phosphatase and specific probes [16], it was essential to identify the protein on SDS gels. The purified Ins(1,4,5)P_3 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_3 5-phosphatase activity.

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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.