Journal of Biochemistry and Molecular Biology, Vol. 31, No. 3, May 1998, pp. 274–280

JOURNAL OF
Biochemistry
and
Molecular Biology

Rat Duodenal Mucosa Inositol Monophosphatase; Novel Enzyme of Which Properties are Distinct from Brain Enzyme

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Received 16 February 1998

An inositol monophosphatase (IMPase) was purified to homogeneity from rat duodenal mucosa for the first time and its enzymatic properties were investigated. Rat duodenal mucosa peculiarly exhibited the highest IMPase activity among various rat tissues examined. By means of ammonium sulfate precipitation, followed by Q-Sepharose, polylysine agarose, reactive-red agarose column chromatography, Uno-Q FPLC, and Bio-Silect FPLC, duodenal IMPase was purified 223-fold to a specific activity of 13.6 U/mg protein. The molecular mass of the native enzyme was estimated to be 48,000 Da on gel filtration. The subunit molecular mass was determined by SDS-PAGE to be 24,000 Da. These results indicate that duodenal IMPase is a dimeric protein made up of identical subunits. Rat duodenal IMPase has distinct properties from brain IMPase. It has a broad spectrum of substrate specificity and is insensitive to Li^+. Duodenal IMPase does not absolutely require Mg^{2+} for its catalytic activity. Furthermore, duodenal IMPase is less stable to heat than brain enzyme. It is suggested that the rat duodenal mucosa needs a large amount of IMPase whose properties are quite different from that of the brain enzyme.

Keywords: Brain, Inositol monophosphatase, Lithium, Molecular weight, Rat duodenal mucosa.

Introduction

The receptor-mediated action of phospholipase C on phosphoinositides gives rise to inositol mono- and polyphosphates, which are sequentially dephosphorylated to inositol (Berridge, 1984; Abdel-Latif, 1986). Inositol monophosphatase (IMPase, EC 3.1.3.25) catalyzes inositol monophosphates to inositol required for resynthesis of phosphatidylinositol and polyphosphoinositides (Gee *et al.*, 1988; Majerus, 1992). This is a crucial step in the mechanism of recycling, since all the pathways within the inositol lipid cycle, as well as the *de novo* synthesis of inositol 1-phosphate from glucose 6-phosphate converge at this point to replenish the pool of free inositol (Mauck *et al.*, 1980).

The enzymes from bovine brain (Hallcher and Sherman, 1980; Attwood *et al.*, 1988; Gee *et al.*, 1988; Meek *et al.*, 1988), porcine brain (Kwon *et al.*, 1993; Kwok *et al.*, 1995), rat brain (Takimoto *et al.*, 1985), and rat testes (Parthasarathy *et al.*, 1993) have been purified and characterized with respect to their physical properties and substrate specificity. They require Mg^{2+} as cofactor and are uncompetitively inhibited by Li^+. Lithium salts which are commonly used in various psychiatric illnesses significantly inhibit IMPase and the inhibition of this phosphatase has been implicated as a therapeutic site of action of Li^+ in manic-depressive disorder (Hallcher and Sherman, 1980; Berridge *et al.*, 1989). Such inhibition results in an increase of inositol monophosphates and a depletion of cellular inositol, possibly controlling the production of inositol lipid precursors involved in signal transduction (Sherman *et al.*, 1981).

On the other hand, inositol has been shown to be involved in the cellular proliferation and development especially during early organogenesis (Akashi *et al.*, 1991). It has been shown that uptake of inositol into cells is associated with parallel changes in cellular proliferation induced by growth factors and hormones (Spizz and Pike, 1992; Grafton *et al.*, 1995). The duodenum is an important organ for digestive functions such as transport, enzymatic digestion, and absorption of nutrients. It is generally

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accepted that the proliferous activity of intestinal epithelial cells is very high. Starting at the base of the mucosa crypts, immature epithelial cells migrate up to the villi, differentiating as they move toward the bowel lumen (Cheng and Leblond, 1974). This process takes 2 to 3 days in rats (Lesher *et al.*, 1961). It is therefore possible that IMPase may be involved in the proliferation of intestinal epithelial cells through generating free inositol. However, intestinal IMPase has never yet been studied.

Thus, we attempted to investigate the duodenal IMPase. In this study, rat duodenal IMPase was purified to homogeneity for the first time and its enzymatic and physicochemical properties were investigated.

Materials and Methods

Materials Inositol 1-phosphate, β-glycerophosphate, adenosine 5′-monophosphate, Trizma base, dithiothreitol, lithium chloride, malachite green, polyvinyl alcohol, SDS-PAGE molecular weight standard, Q-Sepharose, polylysine agarose, reactive-red agarose were purchased from Sigma Chemical Co. (St. Louis, USA). Gel filtration molecular weight standard, Uno Q-6 column, Bio-Silect (SEC 250) column, acrylamide, N,N′-methylene-bis-acrylamide, ammonium persulfate were purchased from Bio-Rad Lab. (Richmond, USA). Ammonium molybdate, p-nitrophenyl phosphate were purchased from Boehringer Mannheim (Indianapolis, USA). All other chemicals were of the highest analytical grade available. Sprague-Dawley rats weighing about 250 g were supplied from the experimental animal center at Hallym University.

Enzyme assay Inositol monophosphatase (IMPase) activity was measured by colorimetric determination of inorganic phosphate (Veldhoven and Mannaerts, 1987) released from hydrolysis of inositol 1-phosphate or β-glycerophosphate (Meek *et al.*, 1988). One unit of enzyme activity was defined as the amount of enzyme converting 1 μmol of β-glycerophosphate into product per 1 min at 37°C. The K_m value of the purified enzyme was determined from the Lineweaver-Burk plot. The protein concentration was estimated by the Bradford procedure using bovine serum albumin as a standard (Bradford, 1976).

Tissue distribution of IMPase Various organs were collected from 7 adult Sprague-Dawley rats and placed on ice. Tissues were diced into small pieces and homogenized in 10 vol (w/v) of 50 mM Tris-HCl, pH 7.4, containing 0.1 mM EDTA and 150 mM KCl using a Polytron tissue homogenizer. Following centrifugation at 20,000 × *g* for 30 min, the supernatants were collected and fully dialyzed three times for 36 h against 10 mM Tris-HCl, pH 7.4, containing 1 mM dithiothreitol (DTT) for the determination of IMPase specific activity.

Purification of rat duodenal IMPase Rat duodenal IMPase was purified to homogeneity by a combination and modification of previously described methods (Meek *et al.*, 1988; Kwon *et al.*, 1993; Parthasarathy *et al.*, 1993). All purification steps were performed at 4°C. The luminal side of the isolated rat duodenum was scraped with a cover glass to obtain a sticky mucosa content. Duodenal mucosa content corresponding to 150 rats (ca. 120 ml) was homogenized in 5 vol (v/v) 50 mM Tris-HCl, pH 7.4, containing 0.1 mM EDTA and 150 mM KCl using a Polytron tissue homogenizer. The homogenate was centrifuged at 20,000 × *g* for 30 min in a refrigerated centrifuge and the pellet was discarded. Solid $(NH_4)_2SO_4$ was slowly added to the stirred supernatant to achieve 30~60% saturation. After additional stirring for 1 h, the suspension was centrifuged at 10,000 × *g* for 30 min and the precipitate was dissolved in 30 ml 10 mM Tris-HCl, pH 7.4, containing 1 mM DTT (buffer A) and then dialyzed two times for 24 h against 100 vol of buffer A.

The dialyzed solution was applied to a 4 × 30 cm column of Q-Sepharose equilibrated with buffer A. The column was washed with 200 ml buffer A and then the enzyme was eluted with a 200 ml linear gradient of 0 to 800 mM NaCl in 10 mM bis-Tris acetate, pH 6.0, containing 1 mM DTT (buffer B). The active fractions were combined, concentrated by ultrafiltration on an Amicon YM 10, and applied to a 1.2 × 15 cm polylysine agarose column equilibrated with buffer B. The column was washed with 80 ml buffer B and the enzyme was eluted by using a linear gradient made with 40 ml buffer B and the same volume of buffer B containing 600 mM NaCl. The active fractions were combined, desalted using an Amicon concentrator, and subsequently passed through a 1 × 8 cm column of reactive-red agarose in buffer B. The enzyme, recovered in the flow-through fractions, was then injected into a 1.2 × 5.3 cm Uno Q-6 column equilibrated with buffer B, connected to the Pharmacia/LKB FPLC system, and eluted with a 90 ml gradient from 0 to 500 mM NaCl in buffer B. The active fractions were combined, concentrated using an Amicon concentrator and injected into a 0.8 × 30 cm Bio-Silect column equilibrated with buffer A. The enzyme was eluted with 30 ml buffer A. Active fractions were pooled and stored frozen in small volumes at −70°C.

Purification of rat brain IMPase Rat brain IMPase has been purified homogeneously and characterized (Takimoto *et al.*, 1985). In the present study, rat brain IMPase was partially purified to compare enzymatic properties with duodenal enzyme. This enzyme was purified by using a $(NH_4)_2SO_4$ precipitation followed by Q-Sepharose, polylysine agarose column chromatography and Bio-Silect FPLC according to the same method described above.

SDS-PAGE and gel filtration SDS-PAGE was performed in 12% polyacrylamide gel by the method of Laemmli (1970). Protein bands and molecular weight standards were visualized by staining with Coomassie blue R-250. The molecular weight of the native enzyme was determined from FPLC on a Bio-Silect gel filtration column with molecular weight standard.

Analysis of data All values were expressed as means ± SD. Statistical analysis was evaluated by the Student's *t*-test. The difference was considered significant if the p value is less than 0.05.

Results and Discussion

Tissue distribution of IMPase As a ubiquitous enzyme involved in the phosphatidylinositol signaling system, IMPase was widely distributed in rat tissues. The relative IMPase activities were duodenal mucosa ≫ ileal

mucosa > pancreas > kidney > brain > lung > stomach as shown in Fig. 1. In the intestine, the mucosa layers contained a significantly higher activity in comparison to the muscle layers. IMPase activity of the duodenal mucosa was 82.1 mU/mg protein, which was 13 times higher than that of the brain. It is very interesting that intestinal mucosa is one of the richest sources of this enzyme. A distinctive feature of the intestinal epithelial cells is its high proliferous activity. Intestinal epithelial cell renewal in rodents occur within every 3 days in the basal layer (Lesher *et al.*, 1961). Because inositol has been known to be involved in cellular proliferation and development (Akashi *et al.*, 1991; Spizz and Pike, 1992), it is possible to speculate that duodenal IMPase may be involved in the proliferation of intestinal epithelial cell through generating free inositol. However, involvement of IMPase in the epithelial cell growth is entirely unknown at the present time and remains to be answered.

Purification of enzyme The initial stages of purification were based on the methods of Meek *et al.* (1988) for the porcine brain enzyme, except that we did not use heat treatment because duodenal enzyme was heat-sensitive. The dialyzed ammonium sulfate precipitate was sequentially subjected to Q-Sepharose, polylysine agarose, and reactive-red agarose column chromatography. The polylysine agarose column chromatography which have been used for the purification of rat testes IMPase by Parthasarathy *et al.* (1993) effectively removed several impurities and, therefore, increased enzyme purity about 10-fold. About 50% of remaining impurities was removed by retention with the reactive-red agarose resin. However, flow-through fractions of the reactive-red agarose column still contained several contaminants. Further purification was accomplished by the use of Uno-Q FPLC and Bio-Silect FPLC. After performing gel filtration FPLC, duodenal IMPase was eluted in a homogeneous form as demonstrated in a SDS-PAGE (Fig. 2). Overall, the enzyme was purified 223-fold over the crude extract with a yield of 6.2%. The specific activity of the purified enzyme was 13.6 U/mg protein (Table 1).

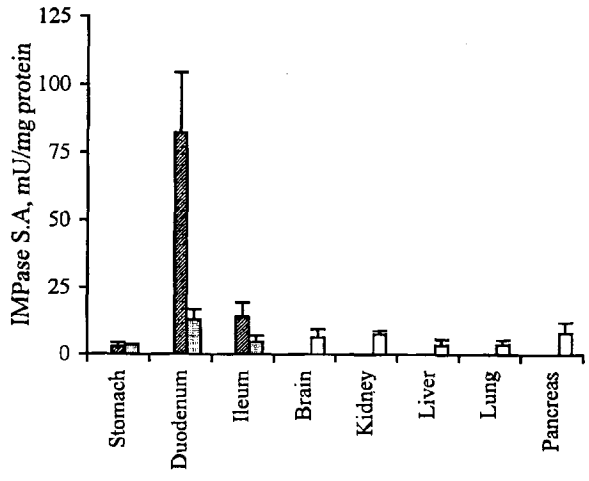

Fig. 1. Tissue distribution of inositol monophosphatase (IMPase) activity in rat. Values are represented as means ± SD of IMPase specific activity in the various tissue homogenates from seven rats. The hatched and dotted bars represent mucosa and muscle layers of the tissue, respectively.

Determination of molecular weight As shown in Fig. 2, SDS-PAGE of the purified duodenal IMPase gave a single protein band. By comparison with the migrations of standard proteins, the molecular weight of the subunit was estimated to be 24,000 Da which was lower than the 29,000 Da reported previously for the rat brain, rat testes, and porcine brain enzymes, respectively (Takimoto *et al.*, 1985; Parthasarathy *et al.*, 1993; Kwok *et al.*, 1995). When

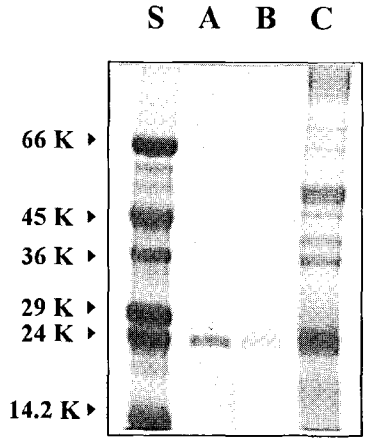

Fig. 2. SDS-PAGE of inositol monophosphatase purified from rat duodenal mucosa. Protein bands were stained with Coomassie Blue R-250. S; Molecular weight standards (bovine serum albumin, 66,000; egg albumin, 45,000; glyceraldehyde 3-phosphate dehydrogenase, 36,000; carbonic anhydrase, 29,000; trypsinogen, 24,000; α-lactalbumin, 14,200 Da); A, Bio-Silect FPLC fraction; B, Uno-Q FPLC fraction; C, Q-Sepharose column fraction.

Table 1. The purification scheme of inositol monophosphatase from 150 rat duodenal mucosa. One unit of activity represents 1 μmol of hydrolyzed substrate/min at 37°C with 1.67 mM β-glycerophosphate.

Step	Protein (mg)	Activity (U)	Specific activity (U/mg)	Purification (fold)
1. Crude extract	2526	154.2	0.061	1.0
2. $(NH_4)_2SO_4$ fractionation	577	71.3	0.124	2.0
3. Q-Sepharose	105	36.7	0.350	5.7
4. Polylysine agarose	8.10	24.5	3.025	49.6
5. Reactive-red agarose	3.62	16.1	4.448	72.9
6. Uno-Q FPLC	1.85	12.3	6.649	109.0
7. Bio-Silect FPLC	0.72	9.8	13.611	223.1

compared to the elution volume of standard proteins on Bio-Silect (SEC-250) FPLC column, duodenal IMPase was eluted in a single peak between bovine serum albumin (66,000 Da) and ovalbumin (44,000 Da), and estimated the native molecular mass to be 48,000 Da (Fig. 3). These results indicate that the native rat duodenal IMPase is composed of two identical subunits. The estimated molecular mass of the native enzyme was also different from those of rat brain (55,000 Da), rat testes (58,000 Da), beef brain (46,000 Da), and porcine brain IMPase (58,000 Da) reported previously (Takimoto *et al.*, 1985; Meek *et al.*, 1988; Parthasarathy *et al.*, 1993; Kwok *et al.*, 1995). A better understanding of the correct size of duodenal IMPase awaits sequence analysis.

Substrate specificity and dependency on magnesium

Both rat IMPases purified respectively from duodenal mucosa and brain hydrolyzed D,L-*myo*-inositol 1-phosphate, β-glycerophosphate, adenosine 5′-monophosphate (AMP), and a general phosphatase substrate p-nitrophenyl phosphate (pNPP), as shown in Fig. 4. The reaction rates of brain enzyme with β-glycerophosphate, AMP, and pNPP were 41%, 2%, and 13% at pH 7.4, respectively, relative to that of D,L-*myo*-inositol 1-phosphate which is the primary receptor-mediated hydrolyzed product. On the other hand, duodenal IMPase hydrolyzed these substrates at higher reaction rates which were 84%, 44%, and 63%, respectively, relative to that of D,L-*myo*-inositol 1-phosphate. It therefore indicates

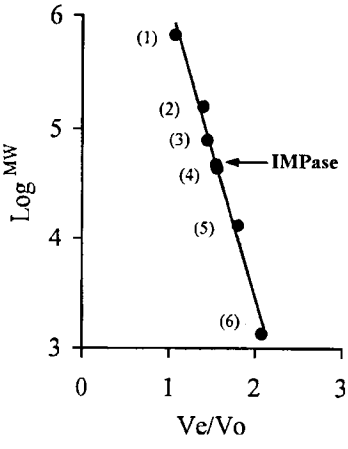

Fig. 3. Molecular weight determination of duodenal inositol monophosphase. The purified enzyme was applied to the Bio-Silect FPLC column (0.8 × 30 cm) equilibrated with 10 mM Tris-HCl, pH 7.4, containing 1 mM DTT, 150 mM NaCl. Calibration was performed with the following standards (1) thyroglobulin (670,000 Da), (2) γ-globulin (158,000 Da), (3) bovine serum albumin (66,000 Da), (4) ovalbumin (44,000 Da), (5) myoglobin (17,000 Da) and (6) vitamin B_{12} (1,350 Da). Void volume of the column was estimated with blue dextran. The elution position of the purified enzyme was indicated by an arrow and a molecular mass of 48,000 Da was estimated.

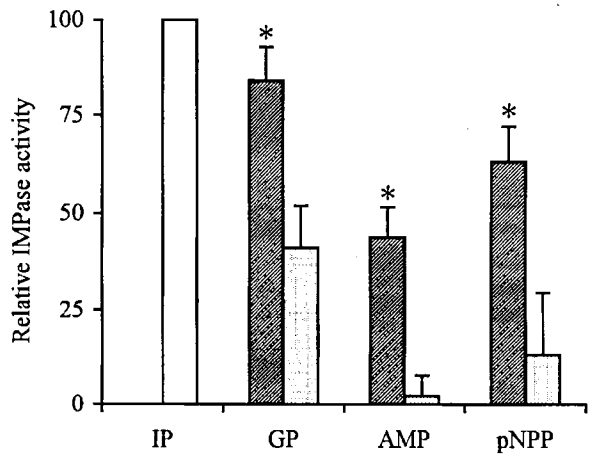

Fig. 4. Substrate specificity of rat inositol monophosphatase purified from duodenum and brain, respectively. The enzyme activity was measured with several substrates (IP, inositol 1-phosphate; GP, β-glycerophosphate; AMP, adenosine 5′-monophosphate; pNPP, p-nitrophenyl phosphate) at a concentration of 1.67 mM under standard assay condition. Values are represented as means ± SD of relative enzyme activity. The hatched and dotted bars represent duodenal and brain enzymes, respectively. Asterisks indicate that the value of the duodenal enzyme is significantly higher than that of the brain enzyme.

that duodenal IMPase has a broader spectrum of substrate specificity than brain enzyme. This feature of the duodenal IMPase was also different from other brain enzymes described previously (Hallcher and Sherman, 1980; Takimoto *et al.*, 1985; Gee *et al.*, 1988). The K_m values of the duodenal enzyme for inositol 1-phosphate and β-glycerophosphate were 0.15 mM and 0.20 mM, respectively. In the case of brain enzyme, K_m values for inositol 1-phosphate and β-glycerophosphate were 0.09 mM and 0.34 mM, respectively.

Mg^{2+} has been known to be a most effective cation to stimulate IMPase activity (Takimoto *et al.*, 1985; Gee *et al.*, 1988; Parthasarathy *et al.*, 1993). We examined the Mg^{2+}-dependence of duodenal and brain IMPases in this study. As shown in Table 2, IMPase activity was increased in a Mg^{2+} dependent manner. However, the catalytic activity of duodenal IMPase was less dependent on Mg^{2+} than the brain enzyme. In the absence of Mg^{2+}, brain IMPase showed faint activity, while duodenal IMPase retained a considerable activity (48% of 5 mM Mg^{2+}). It therefore suggests that the duodenal IMPase does not absolutely require Mg^{2+} for its catalytic activity. Furthermore, brain IMPase activity was slightly inhibited at high concentrations of Mg^{2+} (>10 mM), while no inhibition of duodenal IMPase activity was seen up to 50 mM Mg^{2+}. The Mg^{2+} dependence cannot be explained by the binding of Mg^{2+} to the substrate since only negligible amounts of substrate would have been Mg^{2+}-bound (Hallcher and Sherman, 1980). As suggested for the catalytic mechanism of other phosphatases (Welsh and

Cooperman, 1984; Sowadski *et al.*, 1985), Mg^{2+} could activate the enzyme in different ways. It could acidify water or an active site residue, stabilizing the corresponding base for a nucleophilic attack on the phosphate. Alternatively, it could lower the activation energy of a pentacoordinate transition state by binding to one of the phosphate oxygens. Attwood *et al.* (1988) suggested a proton bridge between a phosphate oxygen and one of the adjacent hydroxyl groups. Further kinetic studies such as solvent isotope effects will be necessary to elucidate the reaction mechanism of Mg^{2+} activation on duodenal IMPase.

Inactivation of IMPase by lithium An interesting feature of IMPase is its inhibition by lithium ions at concentrations which are considered therapeutic in the treatment of manic-depressive illness (Berridge *et al.*, 1989). The purified mammalian IMPases from bovine brain (Hallcher and Sherman, 1980; Gee *et al.*, 1988; Leech *et al.*, 1993), porcine brain (Kwon *et al.*, 1993; Kwok *et al.*, 1995), rat brain (Takimoto *et al.*, 1985), and rat testes (Parthasarathy *et al.*, 1993) are inhibited non-competitively by Li^+. In this study, the ability of Li^+ to inhibit duodenal IMPase activity was examined and compared with that of the brain enzyme. As shown in Fig. 5, brain IMPase was inhibited 56% by 10 mM Li^+, and 72% by 100 mM Li^+. This is in a good agreement with the previously reported observations (Hallcher and Sherman, 1980; Takimoto *et al.*, 1985; Kwon *et al.*, 1993). Contrary to the brain enzyme, duodenal IMPase was hardly inhibited by high concentrations of Li^+. The duodenal enzyme was inhibited 27% by 100 mM Li^+, but not by the lower concentration of 10 mM Li^+ which is covered in the therapeutic concentration range (0.5~1.5 mM) of Li^+ in bovine brain (Hallcher and Sherman, 1980). It indicates that duodenal IMPase is intrinsically insensitive to Li^+ in comparison to the brain enzyme. The lack of inhibition by a lower concentration of 10 mM Li^+ is the most interesting feature of the duodenal IMPase because it suggests that the structural properties of the catalytic domain of the duodenal enzyme may be different from the brain enzyme. A kinetic mechanism for the inhibition by Li^+ has been suggested, in that Li^+ can bind to the species E · inositol · Pi and E · Pi, but not to enzyme/substrate (E · inositol-Pi) complexes (Leech *et al.*, 1993). The cDNA clones encoding the rat brain enzyme and deduced amino acid sequence have already been reported (McAllister *et al.*, 1992). Therefore, it would be worthwhile to clone the genes for rat duodenal enzyme and investigate the structural homology of the catalytic domain with the brain enzyme.

Table 2. Magnesium ion dependency of inositol monophosphatase. The activity was measured in the presence of various concentrations of $MgCl_2$ under the standard assay condition. Values are represented as means ± SD of relative enzyme activity.

[Mg^{2+}] mM	Relative enzyme activity (% of 5 mM Mg^{2+})	
	Duodenal enzyme	Brain enzyme
0	47.6 ± 1.6	15.6 ± 0.6
0.1	53.6 ± 1.3	20.4 ± 0.5
1.0	81.7 ± 2.6	50.7 ± 3.9
1.7	91.1 ± 2.4	101.1 ± 3.9
10.0	110.1 ± 6.0	100.8 ± 3.2
50.0	112.6 ± 1.6	82.3 ± 3.9

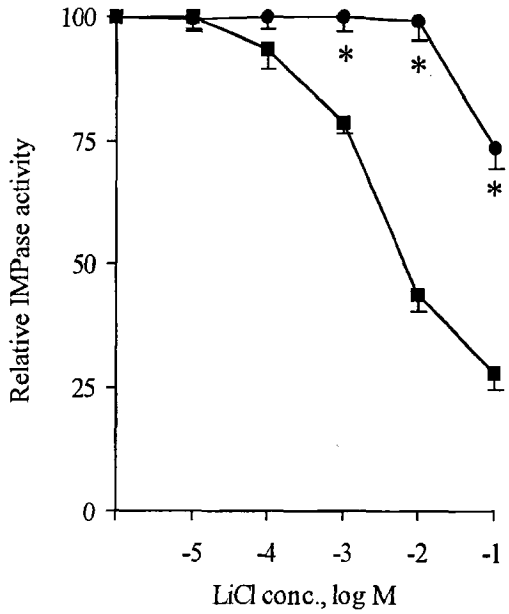

Fig. 5. Inhibition of inositol monophosphatase activity by LiCl. The activity was measured in the presence of various concentrations of LiCl under standard assay conditions. Values are represented as means ± SD of relative enzyme activity. The circle and square represent duodenal and brain enzymes, respectively. Asterisks indicate that the value is significantly higher than the value of the brain enzyme.

Heat stability The brain IMPase is generally known to be a heat-stable enzyme. This remarkable heat resistance has provided the basis for a very convenient means of enzyme purification in most cases (Hallcher and Sherman, 1980; Meek *et al.*, 1988; Kwok *et al.*, 1995). In this regard, the thermal stability of duodenal IMPase was examined and compared with that of brain enzyme. The purified enzymes were incubated in a water bath at 45°C and 75°C for 10~120 min. After rapid cooling in an ice bath, the heat-treated samples were added to a standard assay mixture to measure the enzyme activity. Figure 6 shows the time-dependent inactivation of enzyme activity after heat pretreatment. The brain IMPase did not lose any activity at 45°C for up to 120 min and was partially inactivated at 75°C. The brain enzymes retained 67% and 52% of their activity on heating at 75°C for 10 min and 30 min, respectively. Similar results were reported that rat

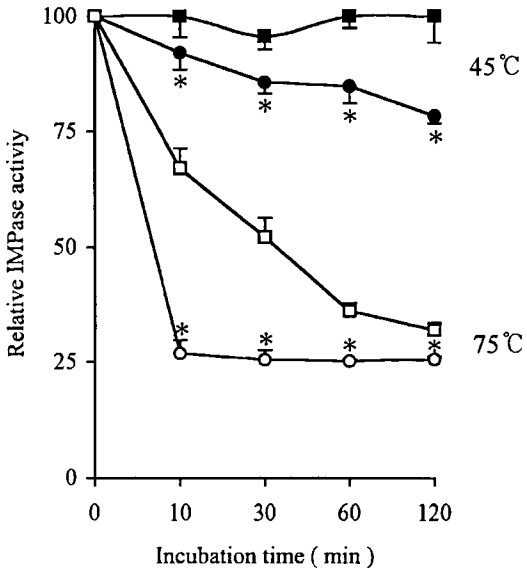

Fig. 6. Time-dependent inactivation of inositol monophosphatase activity after pretreatment of heat. The purified enzyme was incubated in a water bath at 45°C and 75°C for 10~120 min, respectively. After rapid cooling in an ice bath, the heat-treated samples were added to a standard assay mixture. Values are represented as means ± SD of relative enzyme activity. The circles and squares represent duodenal and brain enzymes, respectively. Asterisks indicate that the value of the duodenal enzyme is significantly lower than the value of the brain enzyme.

brain IMPase kept 50% activity on incubation at 70°C for 5 min and porcine brain IMPase retained 75% activity on heating at 70°C for 15 min (Naccarato *et al.*, 1974; Hallcher and Sherman, 1980). Contrary to the brain enzyme, duodenal IMPase was unstable upon heat treatment. The duodenal IMPase gradually lost its activity upon increasing the incubation time at 45°C but still kept 78% activity on incubation for 120 min. At high temperature, the duodenal enzyme was rapidly inactivated and retained 27% activity on heating at 75°C for 10 min. These results indicate that the duodenal IMPase is more sensitive to heat than the brain enzyme and suggests that heat stability is not a general characteristic of IMPase.

In summary, an IMPase was purified to homogeneity from rat duodenal mucosa for the first time and its characteristics compared with rat brain IMPase. The duodenal IMPase seems to be different from the brain enzyme described previously (Hallcher and Sherman, 1980; Takimoto *et al.*, 1985; Gee *et al.*, 1988; Meek *et al.*, 1988; Kwok *et al.*, 1995) with respect to molecular weight, substrate specificity, magnesium ion dependence, lithium sensitivity, and heat stability.

Acknowledgments This research was supported by a grant for basic medical science from the Ministry of Education, Republic of Korea in 1997 and partly by a research grant from Hallym University in 1994.

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