An Automated High Volume Assay to Screen for Inhibitors of myo-Inositol Monophosphatase from Microbial Fermentation Broths

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myo-Inositol monophosphatase (EC 3.1.3.25, IMPase) plays an important role in the phosphoinositide (PI) cell signalling pathway. The enzyme is responsible for the hydrolysis of inositol-1-phosphate, inositol-3-phosphate, and inositol-4-phosphate to generate inositol which is required for the resynthesis of phosphatidylinositol, 1,4,5, triphosphate (1,4,5-IP3) and diacylglycerol (DAG), second messengers mobilising intracellular calcium stores and activation of protein kinase C. In addition, to controlling the recycling of inositol from inositol polyphosphates, IMPase is also a key enzyme in the de novo synthesis of inositol produced by dephosphorylation of Ins (3) P derived from glucose-6-phosphate. Maintenance of the PI signalling transduction pathway therefore depends on the availability of inositol. In most cells uptake of extracellular inositol can be used to replenish depleted intracellular inositol levels, however, in neurons the uptake mechanism is extremely limited. Consequently, IMPase constitutes the major means to replenish inositol levels in neurons and to maintain the PI transduction pathway. One feature of particular note in the catabolic pathway for 1,4,5-triphosphate (1,4,5-IP3) is that two key enzymes, inositol polyphosphate 1-phosphatase and IMPase, are specifically inhibited by lithium ions in the millimolar range. This biochemical event has been proposed to be the underlying mechanism of action of lithium in the therapy of manic-depressive illness. In particular, inhibition of IMPase by lithium has been suggested to lead to depletion of inositol levels in neurons and a consequent attenuation of the PI-linked neurotransmitters. Indeed, lithium leads to accumulation of inositol monophosphates both in vivo and in cells in vitro.

However, despite its remarkable therapeutic properties a severe spectrum of side-effects detract from the therapeutic use of lithium. Furthermore, lithium has other actions, unrelated to IMPase inhibition, on signal transduction pathways and neurotransmission. In this context, development of potent and specific inhibitors of IMPase could lead to new and less toxic drugs for the treatment of manic-depressive illness. In this report we now describe a high volume assay which can be used to detect IMPase inhibitors from microbial fermentation broths.

Assay

IMPase can utilize several substrates including β-glycerolphosphate and hydrolysis of β-glycerolphosphate by IMPase results in the production of free inorganic phosphate which can subsequently be used to monitor enzyme activity. Numerous methods exist for the quantitative determination of phosphate, for example, reaction with molybdate in the presence of Triton X-100 and spectrophotometric reading at 380 nm or 350 nm. In preliminary experiments we determined that the phosphate reaction monitored at 405 nm resulted in a lower background when compared with 350 nm and consequently all further experiments described were performed at this wavelength.

Recombinant human IMPase was expressed in Escherichia coli cells (Pelton et al., in preparation) and was isolated as described previously. The activity of the purified enzyme used in all experiments described was about 15 μmoles phosphate/minute/mg protein. The assay was performed as described by Atwood et al. in buffer A consisting of 50 mM Tris-HCl pH 7.5, 2 mM MgCl2, 0.1 mM EGTA. Purified IMPase was added to buffer and the reaction was initiated by addition of 5 mM β-glycerolphosphate. The reaction was terminated by addition of color mix (0.5% ammonium molybdate, 1% triton X-100, 4.5% H2SO4, in water) and the absorbance was read at 405 nm.

A 96-well microtiter format high volume assay was developed for use with a Beckman Biomek-SL 100 robotic system. In the final conditions selected for the assay 10 μl of either buffer A (control), or known inhibitor (control), or sample to be tested was added to 65 μl of buffer A containing IMPase. The reaction was initiated by addition of 25 μl of β-glycerolphosphate and, after termination by addition of 100 μl of color mix, the free inorganic phosphate was determined by absorbance determination at 405 nm. Preliminary studies showed that color development at 405 nm was linear as a function of the phosphate concentration.

The time-course of phosphate release from β-glycerolphosphate in the presence of IMPase (5 μg/ml) was also examined. As shown in Fig. 1a, the absorbance increase was more or less linear as a function of time for up to 30 minutes, although at longer time periods it tended to deviate from linearity (not shown). The rate of hydrolysis of β-glycerolphosphate as a function of IMPase enzyme concentration was also determined. The substrate was incubated in the presence of different amounts of IMPase (5–30 μg/ml), aliquots were removed at appropriate times, and the reaction terminated by addition of color mix. The initial rate of hydrolysis of β-glycerolphosphate, determined from the linear portion of the curve, was directly proportional to the enzyme concentration (Fig. 1b).
Automation and Data Collection
At low enzyme concentration (5 µg/ml) the rate is linear for time-periods which are suitable for automation on the Biomek SL-100. For four 96-well plates and addition of the various components a 20 minutes time period is required and terminating the reaction after 25 minutes consequently allows a throughput of about 800 samples per hour. These conditions are optimal both for the low amounts of purified enzyme required as well as obtaining an initial rate measurement. The assay was therefore performed on a Biomek SL-100 either in the absence or in the presence of IMPase to determine the standard errors. As shown in Fig. 2, a low standard deviation was observed under these conditions and the signal/background was good. The data collected by the PC can be easily transferred to the LIMS management system.

Two known inhibitors of IMPase, namely lithium and L671,776, were used to further validate the assay and to determine its sensitivity under these assay conditions. IMPase was inhibited in a dose dependent manner by both compounds and approximately 1.25 mM lithium and 450 µM L671,776 were required to inhibit the enzyme by 50% (these values are similar to those reported in the literature[8]).

Natural Product Screening
Preliminary experiments using fermentation broths showed that they frequently interfered in this assay. The major interferences included (1) the presence of significant amounts of phosphate resulting in high background absorbance, (2) the presence of phosphatases which hydrolysed the substrate in the absence of IMPase, and (3) the presence of proteases which degraded IMPase as judged by SDS-PAGE. The broths therefore cannot
be used directly in high throughput screening but require a pre-treatment step. Suitable pre-treatment of broths to eliminate phosphate, phosphatases, and proteases include loading on to a mixed bed resin and elution of bound material with an organic solvent, e.g., ethanol or alternatively a direct solvent extraction. An additional interference which may be encountered in fermentation broths is the presence of chromophores which absorb at 405 nm but can be limited by performing a control wherein the broths are incubated under identical conditions but in the absence of IMPase (the presence of phosphatases can also be easily identified in this manner). Data for 913 fermentation samples showed a variation of 3.1%, suggesting that the assay is robust and does not suffer from non-specific interferences.

Finally, as purification of inhibitors from fermentation broths requires the use of different organic solvents it was important to determine the solvent toleration in the assay. As shown in Table 1 four different organic solvents did not interfere when they constituted up to 10% of the volume in the assay, i.e., the highest concentration at which they could be employed. The assay can therefore be used to routinely follow all purification steps without any requirement to remove organic solvents.

In our screening program this assay was used to screen fermentation samples derived from 12,000 different soil microorganisms. These soil microorganisms included about 4000 fungi, 3000 streptomyces, and 4000 rare actinos. Only six strains produced molecules with an inhibitory activity against IMPase and all six strains were identified as fungi belonging to the Stachybotris/Memnoniella class.

Table 1. The effect of solvents on IMPase activity.

<table>
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<tr>
<th>Solvent</th>
<th>% Inhibition of IMPase activity</th>
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<tbody>
<tr>
<td>Methanol</td>
<td>0</td>
</tr>
<tr>
<td>Butanol</td>
<td>0</td>
</tr>
<tr>
<td>Acetonitrile</td>
<td>12.5</td>
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<tr>
<td>Dimethylsulfoxide</td>
<td>5</td>
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Buffer A containing IMPase was incubated with the indicated solvents at a final solvent concentration of 10%. After initiation of the reaction with β-glycerolphosphate and termination by color mix the absorbance at 405 nm was determined and expressed as % inhibition when compared with control in the absence of solvent.

In conclusion we have developed a high throughput automated assay to search for molecules with inhibitory action on IMPase. The assay is specific and selective and exhibits little or no non-specific interference. The assay can be used to screen complex mixtures such as fermentation broths and the lack of interference from organic solvents makes it suitable for following fractionation steps during the purification of any inhibitory molecules (see ref. 9). In addition, this assay may be useful for other enzymes or reactions which rely on detection of inorganic phosphate.

References