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Comparison of the single molecule activity distributions of recombinant and non-recombinant bovine intestinal alkaline phosphatase

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ABSTRACT

Single molecule assays were performed on bovine intestinal alkaline phosphatase and the recombinant enzyme expressed in *Pichia pastoris* using a capillary electrophoresis-based method. The catalytic rates for the bovine and recombinant enzymes were found to be 11,000 ± 7000 min^{-1} ($N = 161$) and 12,000 ± 7000 min^{-1} ($N = 173$), respectively. Mean catalytic rates and variances did not differ significantly between the enzyme from both sources. Furthermore, the distribution of catalytic rates were indistinguishable.

1. Introduction

Single enzyme molecule assays have shown that individual molecules of a given enzyme have different properties [1–4]. This heterogeneity has been found with respect to catalytic rate, in terms of both V_{max} [5] and K_m [6], activation energy of catalysis [5], electrophoretic mobility [7] and dependence upon exogenous metal ions to maintain activity [8]. Furthermore, catalytic rate has been found to vary for a given molecule over time [9].

Protein expression in the microbial eukaryotic host *Pichia pastoris* allows for the quick production of recombinant proteins in high amounts. *P. pastoris* grows rapidly and to high cell density on inexpensive media. Since it is eukaryotic, *P. pastoris* is able to perform many of the post-translational modifications performed by higher eukaryotic cells. The obtained recombinant proteins undergo protein folding, proteolytic processing, disulfide bond formation and glycosylation [10].

Bovine intestinal alkaline phosphatase is a highly glycosylated enzyme [11]. Single molecule assays have shown this enzyme to be heterogeneous. Differences in glycosylation between individual molecules has been suggested as a possible cause of the observed differences in single molecule catalytic rates [5].

The entire premise of the expression of foreign proteins in *P. pastoris* is that the expressed proteins are identical to that produced in their native organism. This is also the basic assumption used in cell-free expression systems. However, previous studies have shown that the distribution of single molecule activities of *Escherichia coli* β-galactosidase expressed *in vitro* differed from that expressed in *E. coli* [12], the former showing a shift towards lower activity. This was attributed to incomplete removal of the N-terminal methionine or possibly increases in translational errors in the *in vitro* system. This study was undertaken to compare the distribution of single molecule activities of bovine intestinal alkaline phosphatase (bIAP) with the recombinant enzyme expressed with *P. pastoris* (rIAP).

2. Methods and materials

2.1. Reagents

bIAP (product number P5521, affinity purified, 2000–4000 U/mg) and rIAP (product number P8361, ⩾4000 U/mg) were obtained from Sigma–Aldrich. 2′-[2-benzothiazoyl]-6′-hydroxybenzothiazole phosphate (Attophos) and 2′-[2-benzothiazoyl]-6′-hydroxybenzothiazole (Attofluor) were purchased from JBL Scientific. All other reagents were supplied by Sigma–Aldrich.

The assay follows the conversion of the weakly fluorescent substrate Attophos into the highly fluorescent product Attofluor by the actions of the enzyme alkaline phosphatase. Attophos contains some Attofluor which is present as an impurity and must be removed prior to use in order to reduce the background signal in the assay. 20 mM Attophos in 50 mM borate (pH 9.5) was washed with an equal volume of $CHCl_3$ immediately prior to use. 10 μL of

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the washed substrate was added to 485 μL of 40 mM borate (pH 9.5) containing 20% (v/v) DMSO. 5 μL of sufficiently diluted enzyme was added.

2.2. CE instrument

Assays were performed using an in-laboratory constructed capillary electrophoresis instrument equipped with a post-column laser-induced fluorescence detection system [13]. Fig. 1 is a photograph of the instrument. The injection end of a 40 cm long, 5 μm inner diameter fused silica capillary (Polymicro Technologies), along with a platinum electrode connected to a high voltage supply (Spellman model CZE 2000), was placed into a buffer-filled vessel. The applied voltage across the capillary was 16 kV (injection end positive). The detection end of the capillary, from which an approximately 1 mm length of the external polyimide coating was removed by flame, was inserted into a quartz sheath flow cuvette with a 250 by 250 μm inner bore (Hellma). The system was grounded through the sheath flow buffer within the cuvette. A 445 nm, 50 mW laser (Coherent) was used for excitation. Light from the laser was focused with a 6.3×, N.A. 0.2 microscope objective (Melles Griot) approximately 10 μm below the detection end of the capillary. Emission was collected at 90° using a 60×, N.A. 0.7 microscope objective (Universe Kogaku), passed through a 580DF40 optical filter (Omega Optical) and a slit and onto a photomultiplier tube (PMT, Hamamatsu model 1477). The analog PMT signal was collected and digitized using a Pentium 4 computer through a PCI-MIO-16XE I/O board utilizing LabView software (National Instruments) at 10 Hz. The same board was used to control the electrophoresis voltage and PMT bias, which was 1000 V. Sheath buffer was 50 mM borate (pH 9.5) and sample and running buffers were 40 mM borate (pH 9.5) containing 20% (v/v) DMSO.

Sample was injected into the capillary for 210 s at an electric field of 400 V cm^{-1} (injection end positive). The capillary was incubated statically for 30 min at 23 °C. Following this incubation, the capillary contents were mobilized at an electric field of 400 V cm^{-1}.

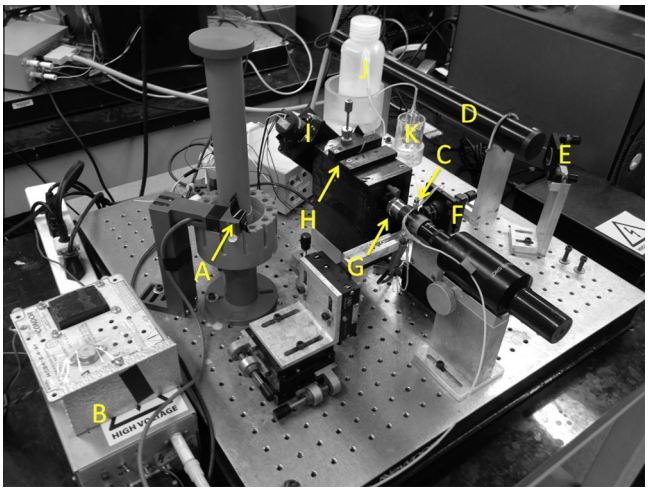

Fig. 1. Photograph of the capillary electrophoresis instrument used. The injection end of the capillary (A) was placed in a vial in the injection carrousel along with a platinum electrode which was connected to a high voltage power supply (B). The detection end of the capillary was placed within a sheath flow cuvette (C). Emission from a laser (D) was redirected with a mirror (E) and focused with a microscope objective (F) onto the cuvette. Emission was collected at 90° using a microscope objective (G), passed through a slit and an optical filter (H) and onto a PMT (I). Sheath buffer flowed by gravity from a reservoir (J), through the cuvette and into a waste reservoir (J).

2.3. Denaturation temperature assay

bIAP and rIAP in10 mM tetraborate buffer (pH 9.3) were incubated at temperatures ranging from 37 to 85 °C for 5 min, immediately followed by colorimetric assay to determine residual activity. The heated sample was diluted 20-fold into 1.0 M diethanolamine (pH 9.8) containing 0.5 mM $MgCl_2$ and 10 mM p-nitrophenylphosphate preheated to 37 °C. The change in absorbance at 405 nm was monitored while the sample was incubated at 37 °C [5].

3. Results and discussion

bIAP and rIAP were both commercially obtained. The supplier of the rIAP stated that they considered their product to be proprietary and were not forthcoming on any details regarding its production. Both samples were analyzed by SDS–PAGE and yielded a single strong band migrating at a nominal mass of approximately 70 kDa, which is consistent with alkaline phosphatase being a homodimer with a molecular mass of 140 kDa (Sigma product information). The rIAP migrated slightly slower than did bIAP. Several additional faint bands were also noted, more so in the bIAP sample, when a very large sample load was used.

bIAP and rIAP were assayed in order to determine their thermal stabilities. The measurements were performed in duplicate. Aliquots of bIAP were incubated for 5 min at 40, 50, 55, 60, 61, 62, 63, 64, 65, 70, 75 and 80 °C and residual activity determined. The temperature at which 50% activity was lost was determined to be 61.5 °C. For rIAP heating was performed at 37, 47, 52, 57, 58, 59, 60, 61, 62, 67, 72 and 82 °C. 50% activity was lost at 60 °C. This indicates that the bIAP and rIAP samples had similar thermal stabilities with the non-recombinant enzyme being slightly more stable.

In the single enzyme molecule assay [5], approximately one third of the 40 cm long capillary was filled with substrate containing very dilute enzyme. Enzyme concentration was such that approximately 4–8 enzyme molecules were present. Since the enzyme molecules were on average 1–3 cm apart and the incubation period of 30 min was relatively short, there was insufficient time for the product formed by one enzyme molecule to diffuse to and mix with that from another. Instead distinct pools of product were formed, each surrounding a given enzyme molecule. Post-incubation these product pools were mobilized past the ultra-sensitive detector, yielding peaks in the resultant electropherogram.

At the concentration used, the substrate produces a substantial signal that would make detection of the small product peaks problematic. It is for this reason that a separation was used. The substrate AttoPhos has the slowest net mobility, the enzyme an intermediate mobility and the AttoFluor product the highest net mobility. Buffer containing substrate and very dilute enzyme was electrokinetically injected into the capillary for 210 s at 400 V cm^{-1}. Although washed with toluene prior, the substrate contains some residual product. The migration time of the product is 470 s and that of the substrate 680 s. After incubation the sample was mobilized at an electric field of 400 V cm^{-1}. This resulted in a 210 s wide plateau in the resultant electropherogram, starting at a migration time of 260 s and ending at 470 s, due to the plug of residual product passing the detector. Pools of product formed by the individual enzyme molecules are seen as peaks sitting atop this plateau. Since the migration time of the substrate is 210 s longer than that of the product, immediately after the product plug emerged the plug of substrate passed the detector for another 210 s from a migration time of 470 to 680 s. This allowed for the detection of the product peaks in the absence of the signal due to