ECONOMIC IMPROVEMENT OF MYO-INOSITOL PHOSPHATE GENERATION BY ENZYME IMMOBILIZATION AND DIRECTED ENZYME EVOLUTION

Ralf Greiner

Department of Food Technology and Bioprocess Engineering, Max Rubner-Institut, Federal Research Institute of Nutrition and Food, Haid-und-Neu-Straße 9, 76131 Karlsruhe, Germany

ABSTRACT

The potential health values of certain mo-inositol phosphates are well known. However, attempts to produce defined isomers of the different partially phosphorylated myo-inositol phosphates by non-enzymatic phytate hydrolysis or chemical synthesis is often associated with high expense and is not very efficient. Enzymatic phytate dephosphorylation was recognized as a cost effective strategy to make pure breakdown products of phytate available in high quantities. In order to improve enzyme stability and facilitate down-stream processing, the phytases were immobilized. Besides enzyme immobilization, directed enzyme evolution was identified as a strategy for an economic improvement of enzymatic myo-inositol phosphate production.

Introduction

Much scientific information has been reported in the last few years linking diet, specific foods, or individual food components with the maintenance of human health and the prevention of chronic diseases such as coronary heart disease, cancer or osteoporosis. Individual myo-inositol phosphate esters were shown to have important physiological functions in man (Shears, 1998). Some of the partially phosphorylated myo-inositol phosphates are involved in the phosphatidylinositol cycle, especially D-myoinositol(1,3,4)trisphosphate and D-myoinositol(1,3,4,5)tetrakisphosphate play important roles as intracellular secondary messengers (Shears, 1998), and some are considered to be pharmaco-active (Carrington et al., 1993; Claxon et al., 1990; Maffucci et al., 2005; Phillippy and Graf, 1997). In addition, dietary myo-inositol phosphates have been suggested to bring about benefits for human health, such as amelioration of heart disease conditions by controlling hypercholesterolemia and atherosclerosis (Jariwalla et al., 1990), protection against diabetes mellitus (Thompson, 1993) and caries (Kaufman and Kleinberg, 1971), prevention of renal stone formation (Jariwalla et al., 2000), and protection against a variety of cancers, in particular colon cancer (Vucenik and Shamsuddin, 2003). So far, the diversity and practical unavailability of individual myo-inositol phosphates preclude their being used.

Access to Individual Partially Phosphorylated myo-Inositol Phosphates

The use of non-enzymatic phytate hydrolysis or chemical synthesis to provide access to individual myo-inositol phosphate isomers is often associated with high expense and is not very efficient. Chemical synthesis is a low yield multi-step process (Plettenburg et al., 2000) and attempts to produce certain partially myo-inositol phosphates have resulted in mixtures of myo-inositolpentakis-(InsP₅), -tetrakis-(InsP₄), -tris-(InsP₃), -bis-(InsP₂) and – monophosphate (InsP) isomers. Purification of these isomers from the mixture is arduous and uneconomical. All in all, 6 different InsP₅, 15 different InsP₄, 20 different InsP₃, 15 different InsP₂ and 6 different InsP exist. The direct production of the desired isomer using enzymes proves far more effective. Phytate was reported to be dephosphorylated by phytases regio- and stereo-selective in a stepwise manner by producing, in general, only one myo-inositol pentakis-, tetrakis-, tris-, bis-, and monophosphate isomer (Konietzny and Greiner, 2002). Phytases (myo-inositol
hexaphosphate phosphohydrolases) [E.C. 3.1.3.8, E.C. 3.1.3.26 and E.C. 3.1.3.72] are a special class of phosphomonoesterases and have attracted considerable attention especially in the animal feed area. Today, phytase is present in about 75% of all diets for simple-stomached animals and its market volume exceeds US$350 million annually (Shivange et al., 2012). However, application of phytase in human foods and medicine can equal, if not exceed, those in animal feeds.

Separation of the different partially phosphorylated myo-inositol phosphate esters was shown to be easily feasible by anion-exchange chromatography. Phytases of different origin may have different phytate dephosphorylation pathways (Greiner and Konietzny, 2011) and the use of different phytases will therefore pave the way to a huge variety of individual partially phosphorylated myo-inositol phosphate isomers. Since the number and distribution of phosphate residues at the myo-inositol ring determine the metabolic effects triggered by the individual myo-inositol phosphate isomers, it is of utmost importance to exploit the full potential of the naturally occurring phytases.

**Immobilization of Phytases**

Immobilisation is one of the efficient methods to improve enzyme stability. Phytases as well as phytase producing cells have been immobilised on a variety of matrices such as NHS-activated Sepharose®, alginate and Fe₃O₄ magnetic nanoparticles as cost-effective bioreactors for large-scale production of these. *Escherichia coli* phytase was covalently immobilized on NHS-activated Sepharose® (Greiner and Konietzny, 1996). The pH dependence of the phytase activity was not influenced by immobilization, whereas stability against heat treatment was enhanced as a consequence of immobilization. Compared to the free phytase the immobilized enzyme exhibits the same excellent substrate specificity, but showed an increased $K_m$-value. The catalytic turnover number ($k_{cat}$) dropped drastically from 6209 s⁻¹ for the free phytase to 1182 s⁻¹ (at 35°C and a flow rate of 5 ml min⁻¹) for the immobilized enzyme. Using the immobilized phytase in a packed-bed bioreactor allows production of individual partially phosphorylated myo-inositol phosphate esters. The maximum amount of these isomers was observed after five passages for D-Ins(1,2,3,4,5)P₅, after nine passages for D-Ins(2,3,4,5)P₄ and after twenty-one passages for D-Ins(2,4,5)P₃. Therefore it is possible to control the amount of the desired degradation product by the number of passages of the phytate solution through the bioreactor.

The purified *Pantoea agglomerans* phytase was entrapped in alginate beads with an entrapment efficiency of >80% (Greiner and Sajidan, 2008). Temperature stability was enhanced as a consequence of entrapment, whereas pH dependence of enzyme activity was not affected. Maximum catalytic activity of entrapped phytase was found at 70°C, whereas the free enzyme exhibited maximal activity at 60°C. Kinetic parameters for hydrolysis of sodium phytate were found to be affected by entrapment. They were determined to be $K_m = 0.84$ mmol l⁻¹ and $k_{cat} = 8$ sec⁻¹ at pH 4.5 and 37°C. Entrapment of *Pantoea agglomerans* phytase was shown to yield longer enzyme lifetime and improved enzyme stability. The entrapped enzyme exhibited the same unique myo-inositol phosphate phosphatase activity as its soluble counterpart. The enzyme hydrolysed only the D-3 phosphate from phytate, producing D-Ins (1,2,4,5,6)P₅ as the sole product of phytate dephosphorylation. Complete conversion of phytate into D-Ins(1,2,4,5,6)P₅ was shown to be feasible by using the enzyme-loaded alginate beads in batch operations. The entrapped enzyme showed a high operational stability by retaining almost full activity even after ten uses.
Phytases of different origin (Escherichia albertii, Aspergillus niger, rye) were covalently bound onto Fe$_3$O$_4$ magnetic nanoparticles (12 nm) via their amino groups (Greiner et al., unpublished data). The high specific surface area of the nanoparticles allows immobilization of a high amount of enzyme per unit mass of the particles. A further advantage of such magnetic particles is the selective separation of the immobilized enzymes from the reaction mixture by application of an external magnetic field. The magnetic nanoparticles were prepared by co-precipitation of Fe$^{2+}$ and Fe$^{3+}$ in an ammonia solution and surface-modified with 2-propene amide-N-(4-methyl-2-butyl-1,3-dioxolan, N,N’-tetramethylene-bis-methacrylamide and N-hexyl methacrylamide to obtain a flexible system in respect to available reactive surface groups. The binding efficiencies of all three phytases were well above 70% relative to the number of aldehyde groups available on the surface of the magnetic nanoparticles. Temperature stability for all three phytases was enhanced as a consequence of immobilization, whereas pH dependence of enzyme activity was not affected. Maximum catalytic activity of the immobilized phytases was found at 60°C (rye), 65°C (A. niger) and 70°C (E. albertii), whereas the free enzymes exhibited maximal activity at 45°C, 55°C and 65°C. The immobilized enzymes exhibited the same excellent substrate specificities and unique myo-inositol phosphate phosphatase activities as their soluble counterparts. However, the catalytic turnover number dropped drastically for the immobilized phytases. The amount of the desired partially phosphorylated myo-inositol phosphate isomer could be easily controlled by the contact time of substrate solution and immobilized enzymes. The immobilized phytases showed a high operational stability by retaining almost full activity even after fifty uses.

Creation of Tailor-made Phytases from Wild-type

Natural occurring enzymes only very rarely meet the requirements given by the industrial processes. To overcome this limitation, tailor-made biocatalysts can be created from wild-type enzymes by protein engineering or by directed evolution techniques. The use of the term “engineering” implies that there is some precise understanding of the system that is being modified. Thus, determinants for the property of an enzyme to be improved must be known and therefore, rational enzyme design usually requires both the availability of the structure of the enzyme and knowledge about the relationships between sequence, structure and catalytic mechanism to make the desired changes. Since site-directed mutagenesis techniques are well-developed, the introduction of directed mutations is easy and relatively inexpensive. The major drawback in rational protein design is that detailed structural knowledge of an enzyme is often unavailable. The relatively few examples where rational protein design has yielded useful enzymes do not negate the view that rational protein design is often a fruitless exercise. In contrast, a collection of methods mimicking the natural process of enzyme evolution in the test tube by using modern molecular biology methods of mutation and recombination provides a powerful tool for the development of biocatalysts with novel properties without requiring knowledge on enzyme structures or catalytic mechanisms. This collection of methods has been termed directed evolution (Chirumamilla et al., 2001). The use of directed evolution techniques has rapidly emerged to be the method of choice for the development and selection of mutated enzymes with improved properties. Directed evolution mimics the process of Darwinian evolution in a test tube combining random mutagenesis and recombination with screening or selection for enzyme variants that have the desired properties. The genetic diversity for evolution is created by mutagenesis and/or recombination of one or more parent sequences. These altered genes are cloned back into a plasmid and functionally expressed in a suitable microbial host, such as Escherichia coli or Saccharomyces cerevisiae. Clones expressing improved enzymes are identified in a high-throughput screen or selection, and the gene(s) encoding those improved
enzymes are isolated and recycled to the next round of directed evolution in order to accumulate beneficial mutations (Arnold and Volkov, 1999). Thus, a directed evolution experiment is “directed” in the sense that the researcher selects the variant enzymes that better meet some criterion via a series of stepwise improvements. The likelihood of success in a directed evolution experiment is directly related to the total library size, as evaluating more mutants increases the chances of finding one with the desired properties. Performing multiple rounds of evolution is useful not only because a new library of mutants is created in each round, but because each new library uses better mutants as templates. This means, the "winners" of the previous round are diversified in the next round to create a new library.

Thus, besides enzyme immobilization, directed enzyme evolution was identified as a strategy for an economic improvement of enzymatic myo-inositol phosphate generation. This strategy was applied to improve temperature stability of the Escherichia albertii phytase (Greiner, unpublished data). Error-prone PCR was performed using a plasmid containing the wild-type Escherichia albertii phytase encoding gene cloned into a Saccharomyces cerevisiae expression vector as a template. Error-prone PCR uses low-fidelity polymerization conditions to introduce a low level of point mutations randomly over a long sequence. Thus, the steps are small, preferably single amino acid substitutions in each generation, and multiple such mutations are accumulated, either sequentially or by recombination (Stemmer, 1994), to acquire the desired function (Arnold, 1998). Approximately, 1500 clones were screened for increased temperature stability. Compared with the wild-type enzyme, two variants (K46E and D144N/V227A) showed a significant increase in temperature stability. Compared to the wild-type phytase, the mutants showed a 33% (K46E) and a 95% (D144N/V227A) higher residual activity at 80°C after 10 min incubation. Overall catalytic efficiency (k/[M]) of K46E and D144N/V227A was improved by 36% and 97% compared to the catalytic efficiency of the wild-type phytase at pH 4.5, respectively. Thus, the catalytic efficiency of these enzymes was not inversely related to their temperature stability. From an economic point of view it is worth mentioning that the mutants still exhibit excellent high specific activities.

Conclusion
Enzymes used in biotechnological applications should be effective, stable to resist inactivation by processing conditions and storage, and cheap to produce. The application of phytases to produce individual partially phosphorylated myo-inositol phosphate isomers for use in human health and medicine may represent an exciting new avenue. However, so far the reuse of phytases was hampered by their lack of stability under processing conditions and difficulties to be recovered from the reaction mixtures. Fortunately, these problems have been overcome by immobilization of the phytases on a variety of matrices. The developed bioreactors could be used to produce individual partially phosphorylated myo-inositol phosphates cost effective in large scale. Furthermore, Escherichia albertii phytase variants with a better overall catalytic efficiency and improved temperature stability were obtained by error-prone PCR. These improvements make these enzymes better suited for the intended biotechnological applications.

References


