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Thermal Stabilization of *Aspergillus* phytase by L-Arginine

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Phytase from *Aspergillus* species is a very heat unstable enzyme which inactivates to a great extent during the thermal processing of animal feed formulation. Various protein stabilization additives were tested to improve its heat stability. Among them, a basic amino acid, L-arginine remarkably increased the thermal stability of phytase in an aqueous solution state.

Key words: phytase, stability, L-arginine, thermal stabilization

INTRODUCTION

Phytase (myo-inositol hexaphosphohydrolase) hydrolyzes phytic acid into myo-inositol and phosphate [1]. The phytic acid which is abundant as a form of phosphate binding storage material in cereals and legumes has caused serious phosphorous pollution problems in monogastric animal livestock production [2, 3]. The low absorption bioavailabilty of phytic acid in animals of pig and poultry as well as its complex formation with essential dietary minerals require the use of phytase in animal feed formulation not only to reduce the release of phosphorous into environment, but also to increase the nutritional values of essential minerals such as calcium, magnesium, and zinc ions [4]. The phytase as an animal feed component is routinely incorporated into compounded feed by a pelleting process which involves a steam heating step followed by an extrusion through a die for making easyhandle feed product. The pelleting process is an exposure of enzyme to the combined harsh conditions of heat (60-90°C) and high humidity for a relatively short period, which severely inactivates the phytase. Thus, it is highly desirable to enhance the thermal staility of phytase to maximize its survival activity in pre-mix feed formulation.

There have been a number of studies on the thermal stabilization of proteins by adding simple chemical additives in biopharmaceutial industry [5]. A basic principle behind the protein stabilization has been understood by preferential hydration mechanism. Most of the additives known to be protein stabilizers are polyols and sugars which generally increase the thermodynamic tendency of protein folding by excluding protein molecules from water environment [6]. In this study, various carbohydrate and other additives were screened and selected to increase the thermal stabilization of phytase in aqueous solution formulation. The optimum formulation recipe was determined based on the results from thermal inactivation results and compared to those of commercially available recombinant phytase products.

Materials and Methods: Phytase from *Aspergillus ficuum*, which is a crude form with 3.5 unit/mg protein at pH 2.5 and 37°C, was obtained from Sigma Co. One commercially available phytase (Maxazyme® FT) which has been used for the steeping of corn wet-milling process was obtained from Gist-brocades Co. Its nominal activity was 5,000 FTU/g. It was diluted to 5 FTU/ml with buffer solution and used. Phytic acid (dodecasodium salt form of inositol phosphoric acid), ammonium heptamolybdate, ammonium vandadate, and other chemicals were all from Sigma Co. The enzymatic activity of phytase was determined by quantifying the liberated phosphorous amount from the substrate by a colorimetric assay [7]. One mg/ml of phytase concentration in 0.1 M acetate buffer (pH 5.5) solution containing 0.1M calcium chloride was used for the stability test. The thermal inactivation experiments were carried out at 60°C, otherwise stated. The enzyme was incubated for various time intervals at 60°C and then cooled to room temperature to assay the remaining activity.

RESULTS AND DISCUSSION

Figure 1 shows the stability profiles of phytase as a function of temperature at pH 5.5. It can be seen that the phytase activity rapidly decreases at 60°C, whereas below 60°C, its initial activity maintains for at least 120 hr. The activity-temperature profile indicated that the optimum temperature for maximum activity was at 55°C. Thus, 60°C is above the denaturation temperature of phytase. To screen various additives to enhance the phytase thermal stability, the following sugars of trehalose, glucose, sucrose, and sorbitol with 5% (w/v) concentration respectively, and one amino acid, L-arginine with 1% (w/v) concentration were added into the enzyme solution, and then tested for their thermal stabilization effects.

Figure 2 shows the effect of various additives on the phytase thermal stabilization at 60°C. All the sugars did not exhibit any stabilization effects on phytase, but L-arginine, a basic amino acid, remarkably increased

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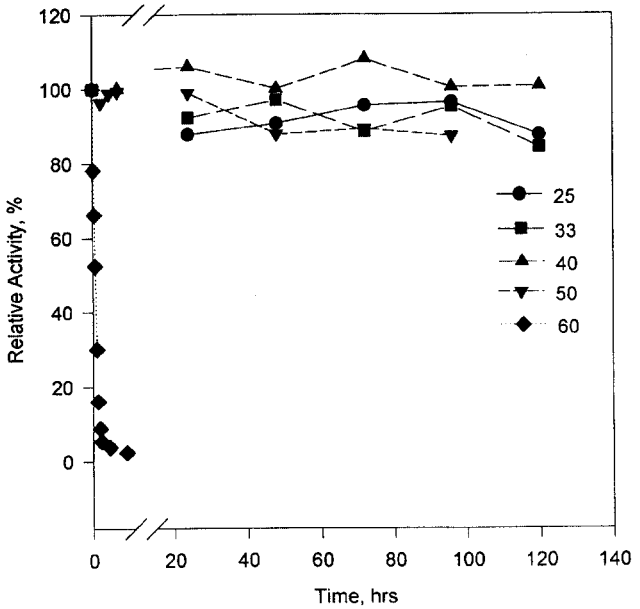

Fig. 1. Stability of phytase at various temperatures as a function of temperature.

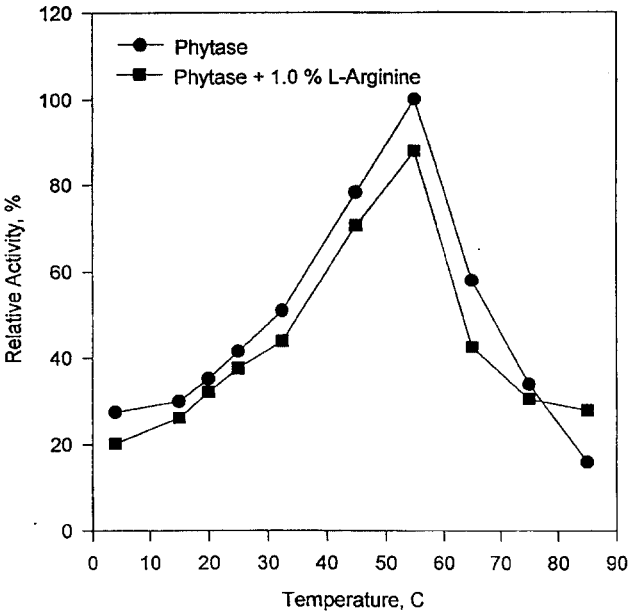

Fig. 3. Activity-temperature profiles of phytase in the presence and absence of L-arginine.

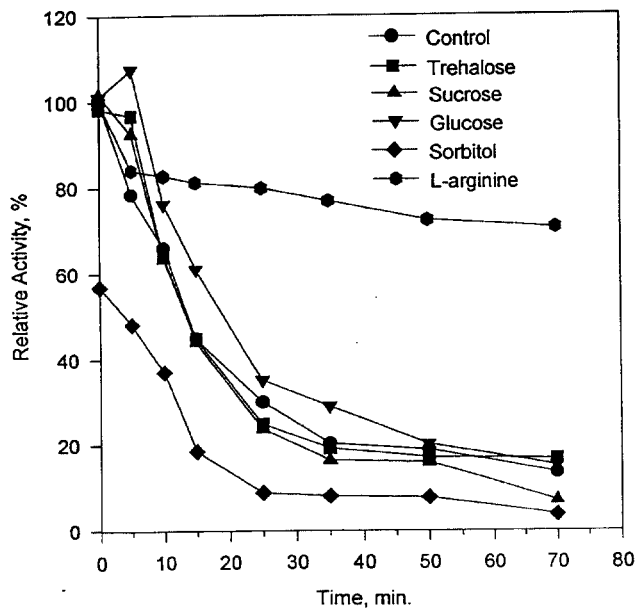

Fig. 2. Effect of various additives on phytase stability at 60°C.

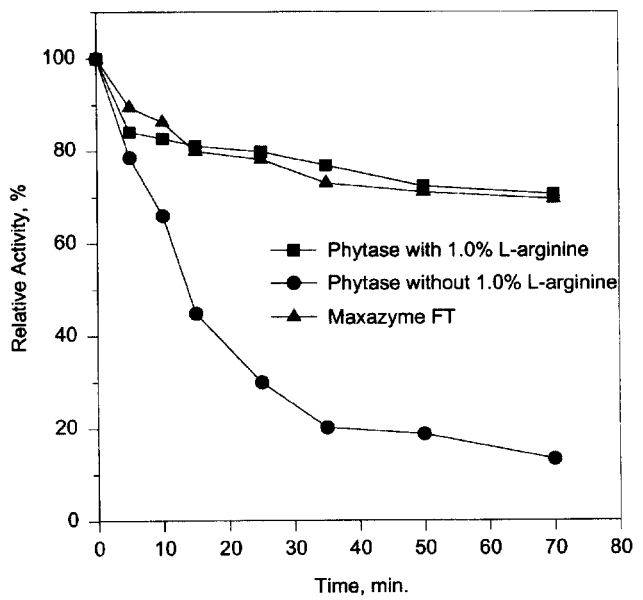

Fig. 4. Comparative phytase stability profiles of phytase formulated with L-arginine and one commercial phytase product.

the phytase stability. Among the sugar additives, sorbitol seems to act as an inhibitor to phytase.

To determine whether L-arginine increase the phytase activity, the phytase activity in the presence of 1% (w/v) L-arginine was determined. Figure 3 shows the comparative phytase activity profiles with and without L- arginine. There is a slight decrease in activity in the presence of L-arginine, indicating that L-arginine acts as a phytase stabilizer, not as a phytase activator. Thus, it can be deduced that L-arginine somehow effectively stabilizes the three dimensional conformation of phytase. The effect of L-arginine concentration on the phytase stability was studied in a separate experiment; it was found that 0.75-1.0% (w/v) L-arginine concentration range in phytase solution exhibited maximum stabilization effect when tested up to 2.0% (w/v) L-arginine concentration.

Figure 4 shows the comparative stabilization effects of crude phytase formulated with L-arginine and one of commercially available phytases that may contain undisclosed proprietary protein stabilizers. It can be seen that the phytase formulation containing L-arginine show comparable stability result to that of the commercial product.

L-arginine has been used as a unique additive for the stabilization of tissue plasminogen activator in a liquid dosage formulation. It has been reported that the addition of L-arginine not only shifted the denaturation temperature of tissue plasminogen activator from 66°C to 71°C, but also prevented the cleavage of peptide bonds [8], resulting in one of successful therapeutic protein formulation cases. Although its exact stabilization mechanism is still unclear, L-arginine has been known

to bind to some regions of protein via ionic interaction with concomitant stabilization of protein structure. In the case of phytase, it is not clear whether the above stabilization mechanism can be applied. Further detailed studies are necessary to elucidate how L-arginine played a role in thermal stabilization of phytase. In summary, L-arginine is a very effective stabilization agent for phytase at higher temperatures which is required for the pelleting process of animal feed formulation.

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