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Improvement of phytase thermostability by using sorghum liquor wastes supplemented with starch

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Abstract

When a phytase solution, soluble starch, and sorghum liquor wastes were mixed at the ratio of 1:1:10 (v/w/w), the residual phytase activities after 30 min of treatment at 70 and 80 °C were respectively, about 90% and 18% of that at 37 °C. After 10 min treatment, the residual activity was 67% at 80 °C and 10% at 90 °C.

Introduction

Enzyme thermostability is very important in industrial applications that often involve high temperature processes. Much research has focused on the isolation of thermostable enzymes from thermophilic or hyperthermophilic microorganisms (Legin *et al.* 1998). Other researchers have tried to create thermostable enzymes (Kumar *et al.* 2000) using UV and chemical mutagenesis or modern molecular biology techniques, such as manipulation or site-directed mutation. By generating vast libraries of mutants, such techniques have led to a huge screening problem. In addition, low enzyme activity or substrate specificity usually makes these enzymes useless. For example, artificially replacing proline (Watanabe & Suzuke 1998) or introducing disulfide bonds (Scott & Steven 2000) in enzymes shows promise for improving the thermostability by decreasing the freedom of the enzyme structure. Unfortunately, it usually requires multiple amino acid replacement (Kumar *et al.* 2000); so while this approach might enhance the thermostability, it also may alter the characteristics of the enzymes themselves.

Another approach is to improve the enzyme thermostability by using additives. Salts and polyols have been used to increase the thermostability of enzymes (Obon *et al.* 1996, Lamosa *et al.* 2000), but the enzyme's activity will be influenced by a high concentration of salts. Although commercial enzymes may possess some thermostability, their specific formulations are usually held in strict confidence. It is our goal to develop an approach that can be universally used to improve enzyme thermostability. Sorghum liquor wastes (SLW) were generated after two distillations from solid-state fermented sorghum. We accidentally detected enzyme activity from the SLW (unpublished data), indicating us that SLW might be able to protect enzymes at high temperatures. Phytase is the major enzyme used by the feed industry which usually needs to the thermostable to withstand high pelleting temperatures. In this paper, we demonstrate that phytase thermostability can be improved by using SLW supplemented with starch.

Materials and methods

Enzyme and chemicals

Commercial phytase, Natuphos, was purchased from BASF (Ludwigshafen, Germany). Other chemicals were from Sigma (St. Louis, MO, USA). Fresh sorghum liquor wastes (SLW) were provided by the Research Institute for Wine, Taiwan Tobacco and

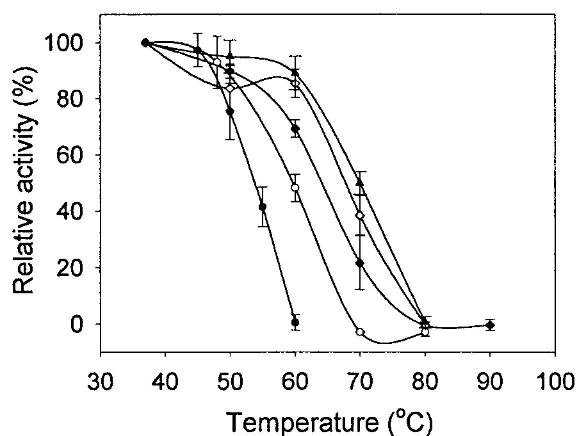

Fig. 1. Effects of sorghum liquor wastes (SLW) on phytase thermostability. Phytase solutions were mixed without (●) or with SLW at the ratio of 1:1 v/w (○); 1:2 (♦); 1:4 (◊); or 1:10 (▲) and treated at different temperatures for 30 min ($n = 3$; error bar = SE). The activity at 37 °C was set as 100% = 1114 U ml^{-1} extract.

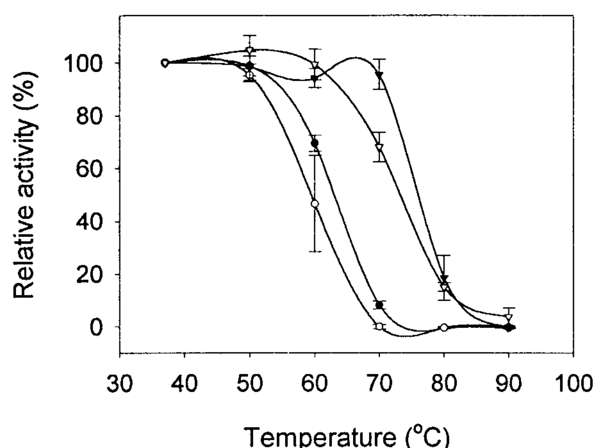

Fig. 2. Effects of sorghum liquor wastes (SLW) supplemented with starch on phytase thermostability. For the experiments without SLW, the phytase solution was mixed with equal amounts of soluble starch (●) or modified starch (○) and treated at different temperatures for 30 min. For the experiments with SLW, phytase, soluble starch (▼) or modified starch (▽), and SLW were mixed at a ratio of 1:1:10 (v/w/w) ($n = 3$; error bar = SE). The activity at 37 °C was set as 100% = 1054 U ml^{-1} extract.

Wine Monopoly Bureau (Taipei, Taiwan). SLW were dried at 60 °C for 24 h, then ground and sieved through 0.64 cm mesh. Soluble starch was purchased from Waco Chemical Industry (Osaka, Japan), and modified starch, Clearam MH-10, was from the Gemfont Corporation (Taipei, Taiwan).

Phytase assay method

Phytase activity was assayed according to Bae *et al.* (1999), and the absorbance at 700 nm was measured by a Versamax microplate reader (Molecular Devices, Sunnyvale, CA). One unit of phytase activity was defined as the amount of activity that releases 1 μmol phosphate per min at 37 °C.

Thermostability assay

Phytase was extracted from 1 g Natuphos powder (5000 U g^{-1}) using 4 ml 100 mM sodium acetate buffer (pH 5) to give 1250 U ml^{-1}. After centrifuging at 12 400 *g* for 10 min, 1 ml of the supernatant was mixed with different amounts of SLW and incubated at 37, 50, 60, 70, 80, or 90 °C for 30 min. The phytase and SLW mixtures were then extracted with 100 mM acetate buffer (pH 5), and the phytase activity was assayed at 37 °C. Similar experiments were carried out using SLW supplemented with soluble starch or modified starch.

Results

Effect of SLW on phytase thermostability

The effects of SLW on phytase thermostability are shown in Figure 1. For the experiments without SLW, about 80% and 40% of activity remained after 30 min of treatment at 50 and 55 °C, respectively. No phytase activity was detected after exposure to 60 °C for 30 min without SLW. When phytase and SLW were mixed at 1:1 (v/w), more than 80% of activity remained after 55 °C treatment, and 50% activity remained after 60 °C treatment. When mixing phytase with SLW at 1:10 (v/w), 90% and 50% of activity remained after treatment at 60 and 70 °C, respectively, while phytase was inactivated at 80 °C.

Effect of SLW supplementation with starch on phytase thermostability

Figure 2 illustrates the effects of SLW supplementation with starch on phytase thermostability. When phytase was mixed with soluble starch at 1:1 (v/w), 70% activity remained after treatment at 60 °C for 30 min, while no activity could be detected at 70 °C. When phytase, soluble starch, and SLW were mixed at 1:1:10 (v/w/w), the residual phytase activity was about 90% after 70 °C treatment, and 18% activity was recovered after 80 °C treatment. Phytase was inactivated when held at 90 °C. Since soluble starch would be

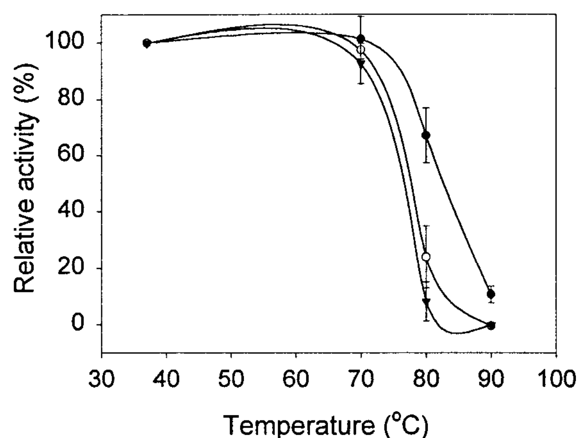

Fig. 3. Effects of heating time on the thermostability of phytase, soluble starch, and SLW mixed at a ratio of 1:1:10 (v/w/w) and heated to different temperatures for 10 (●), 20 (○), and 30 min (▼) (n = 3; error bar = SE). The activity at 37 °C was set as 100% = 1072 U ml^{-1} extract.

dissolved at temperatures over 70 °C, modified starch with higher temperature resistance was used next. The residual phytase activity after 70 °C treatment was 70% using SLW supplemented with modified starch, in comparison with a residual activity of 90% when using soluble starch (Figure 2). However, the activity at 80 °C was similar to that using SLW and soluble starch, and there was about 5% activity detected after treatment at 90 °C. Because the pelleting time is much shorter than 30 min, we reduced the heat treatment time to 10 min, after which we measured residual activities of 67% at 80 °C and 10% at 90 °C (Figure 3).

Discussion

Most feed enzymes are sensitive to hydrothermal processing. Manufacturers usually increase enzyme thermostability by improving either the formulation (coating) or chemical complex. Enzymes adsorbed onto some polymers may change their biochemical characteristics, such as optimal pH and thermostability. Phytases covalently immobilized on Fractogel (Ullah & Cummins 1987) or gelatin (Liu *et al.* 1999) showed a higher optimal temperature than did free phytase, but the catalytic activity was reduced. In this study, we demonstrate that thermostability was improved when phytase was mixed with SLW and starch. The improved thermostability might be attributed to increased heat transfer resistance using SLW, and consequently enzymes are protected from heat. Another possible explanation is that adsorbing onto SLW might prevent the unfolding of enzymes caused by heat treatment. Supplementation of starch might help the adsorption between phytase and SLW. In addition, starch may adsorb water, thus reducing the heat conductivity and improving the thermostability. Although the detailed mechanism still requires further investigation, using SLW seems to provide an alternative approach to improve enzyme thermostability.

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