Effects of Extraction Solution and Solid-Liquid Separation Methods on Solid-State Non-Starch Polysaccharidase Activity: Postprint

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Abstract

This experiment aimed to investigate suitable extraction solutions and solid-liquid separation methods for enzymes in the evaluation of solid-state enzyme preparations. A 4$×$3 two-factor completely randomized design was employed, wherein the extraction solutions were deionized water, acetate buffer (0.1 mol/L, pH 5.50), phosphate buffer (0.05 mol/L, pH 6.00), and 0.9% NaCl solution; the solid-liquid separation methods following solution extraction were no separation, centrifugation at 3,000 r/min for 3 min, and filtration with medium-speed filter paper. Each treatment comprised 5 replicates with 2 parallels per replicate, enzyme activity was measured under each treatment, and the influence of extraction solution type on solute and protein content in the solution after dissolution and centrifugation of enzyme preparation products (except α-galactosidase) was examined. The results demonstrated that xylanase activity was highest after dissolution in phosphate buffer ($P<0.05$); β-glucanase activities after dissolution in acetate buffer, phosphate buffer, and 0.9% NaCl solution were comparable ($P>0.05$), and all were significantly higher than that in deionized water ($P<0.05$); β-mannanase activity was highest after dissolution in deionized water, followed by acetate buffer, both of which were significantly higher than phosphate buffer and 0.9% NaCl solution ($P<0.05$). Extraction solution type exerted no significant effect on α-galactosidase activity ($P>0.05$). The solid-liquid separation method after enzyme preparation dissolution had no significant effect on measured xylanase activity ($P>0.05$); β-glucanase activity was highest after centrifugation or filtration of the extraction solution ($P<0.05$); β-mannanase activity was highest after centrifugation of the extraction solution ($P<0.05$); whereas α-galactosidase activity was highest when the extraction solution was not separated ($P<0.05$). A highly significant interaction existed between extraction solution type and solid-liquid separation method after enzyme preparation dissolution on the measured activities of the four non-starch polysaccharide enzymes

(P<0.01). Acetate buffer exhibited the highest solubility for xylanase preparation (P<0.05), while both deionized water and 0.9% NaCl solution displayed the highest solubility for β-glucanase and β-mannanase preparations (P<0.05). However, acetate buffer yielded the lowest protein content in the extraction solution after dissolving xylanase, β-glucanase, and β-mannanase (P<0.05). These results indicate that acetate buffer can most effectively extract enzyme proteins when dissolving the four solid-state enzyme preparations, solid-liquid separation is not recommended after α-galactosidase extraction, while centrifugation is appropriate for the extraction solutions of the other three enzymes.

Full Text

Effects of Extraction Solution and Solid-Liquid Separation Method on the Activities of Non-Starch Polysaccharide Enzymes

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Abstract

This experiment investigated appropriate extraction solutions and solid-liquid separation methods for evaluating enzyme activities in solid feed enzyme products. A 4$×$3 factorial completely randomized design was employed, with four extraction solutions: deionized water, acetic acid-sodium acetate buffer (0.1 mol/L, pH 5.50), phosphate buffer (0.05 mol/L, pH 6.00), and 0.9% NaCl solution; and three solid-liquid separation methods after extraction: no separation, centrifugation at 3,000 r/min for 3 min, and filtration with medium-speed quantitative filter paper. Each treatment comprised five replicates with two parallel determinations per replicate. Enzyme activities were measured under each treatment condition, and the effects of extraction solution type on solute and protein contents in the supernatant after dissolution and centrifugation were examined (excluding α-galactosidase).

The results demonstrated that phosphate buffer yielded the highest xylanase activity ($P < 0.05$). Acetic acid-sodium acetate buffer, phosphate buffer, and 0.9% NaCl solution produced comparable β-glucanase activities ($P > 0.05$), all significantly higher than deionized water ($P < 0.05$). Deionized water resulted in the highest β-mannanase activity, followed by acetic acid-sodium acetate buffer, both significantly exceeding phosphate buffer and 0.9% NaCl solution ($P < 0.05$). Extraction solution type did not significantly affect α-galactosidase activity ($P > 0.05$). Solid-liquid separation method had no significant effect on xylanase activity ($P > 0.05$), while centrifugation or filtration yielded higher β-glucanase

activity ($P < 0.05$). Centrifugation produced the highest β-mannanase activity ($P < 0.05$), whereas no separation resulted in the highest α-galactosidase activity ($P < 0.05$). A highly significant interaction between extraction solution type and solid-liquid separation method was observed for all four non-starch polysaccharide enzymes ($P < 0.01$). Acetic acid-sodium acetate buffer provided the greatest solubility for xylanase products ($P < 0.05$), while deionized water and 0.9% NaCl solution maximized solubility for β-glucanase and β-mannanase products ($P < 0.05$). However, acetic acid-sodium acetate buffer yielded the lowest protein content in extracts of xylanase, β-glucanase, and β-mannanase ($P < 0.05$). These findings indicate that acetic acid-sodium acetate buffer most effectively extracts enzyme protein from solid enzyme preparations. α-Galactosidase extracts should not undergo solid-liquid separation, whereas the other three enzymes are suitable for centrifugal separation after extraction.

Keywords: non-starch polysaccharide enzymes; extraction solution; solid-liquid separation; enzyme activity

Introduction

Feed non-starch polysaccharide enzyme products in the domestic market predominantly exist as solid formulations where fermentation broth is adsorbed onto carriers. All reactions for evaluating the enzymatic characteristics and hydrolytic effects of solid feed enzyme products occur in liquid phase. Extracting feed enzymes from solid preparations using appropriate solutions is the first critical consideration in enzyme activity assessment, making the selection of suitable extraction solutions and post-extraction solid-liquid separation methods essential. Current assays for feed non-starch polysaccharide enzyme activity typically employ acetic acid-sodium acetate buffer for extraction, followed by centrifugation to separate the enzyme solution from insoluble residues.

In in vitro digestion evaluations of feed enzyme efficacy, two addition methods are used: direct addition to diets, suitable for liquid enzymes in small samples or solid enzymes in batch diet preparation; and extraction of solid enzymes followed by centrifugation and supernatant addition to reaction systems, appropriate for uniformly adding feed enzymes to small feed samples. However, buffer type and solid-liquid separation method both influence measured enzyme activity in solid enzyme preparations. Therefore, investigating optimal extraction solutions and separation methods is crucial for solid enzyme product evaluation. This study examined the effects of extraction solutions and post-extraction solid-liquid separation methods on measured enzyme activities, as well as the influence of extraction solution type on solute and protein contents in dissolved solutions, to identify suitable pretreatment procedures for feed enzyme product assessment.

Materials and Methods

1.1 Feed Enzyme Products

Feed enzyme products were provided by a Beijing biotechnology company, with enzymatic characteristics listed in Table 1.

1.2 Experimental Design

The study consisted of two parts. Experiment 1 investigated the effects of extraction solution and solid-liquid separation method on enzyme activity measurements using a 4$×$3 factorial completely randomized design. The four extraction solutions were deionized water, acetic acid-sodium acetate buffer (0.1 mol/L, pH 5.50), phosphate buffer (0.05 mol/L, pH 6.00), and 0.9% NaCl solution, with a 30-min dissolution time. The three solid-liquid separation methods were no separation, centrifugation at 3,000 r/min for 3 min, and medium-speed quantitative filter paper filtration. Twelve treatments were evaluated, each with five replicates and two parallel determinations, measuring activities of four enzyme preparations.

Based on Experiment 1 results, Experiment 2 further examined the effects of extraction solution type on solute and protein contents in dissolved solutions after centrifugation at 3,000 r/min for 3 min (except for α-galactosidase) to determine impurity levels. Four extraction solutions were tested as in Experiment 1, with five replicates per treatment and two parallel determinations each. In all treatments, solid enzyme preparations were dissolved in extraction solution at a 2:100 mass-to-volume ratio.

1.3 Analytical Methods

1.3.1 Enzyme Activity Determination Xylanase activity was determined according to GB/T 23874–2009, defined as the amount of enzyme required to release 1 mol of reducing sugar per minute from a 5 mg/mL xylan solution (beechwood, Sigma X4252) at 37 °C and pH 5.50. β-Glucanase activity was measured per NY/T 911–2004, defined as the enzyme quantity releasing 1 mol reducing sugar per minute from a 4 mg/mL barley glucan solution (Sigma G6513) at 37 °C and pH 5.50. β-Mannanase activity was assayed using enterprise standard Q/HDTZW 0008–2012, defined as the enzyme amount releasing 1 mol reducing sugar per minute from a 3 mg/mL locust bean gum mannan solution (Sigma G0753) at 37 °C and pH 5.50. α-Galactosidase activity was determined via enterprise standard Q/HDTZW 0009–2012, defined as the enzyme quantity releasing 1 mol p-nitrophenol per minute from 10 mmol/L p-nitrophenyl-α-D-galactopyranoside at 37 °C and pH 5.50.

1.3.2 Solute Content Determination in Extraction Solution Enzyme preparation (0.8 g) was dissolved in 40 mL extraction solution at 0.02 g/mL concentration, stirred on a magnetic stirrer at 4 °C for 30 min, then centrifuged

at 3,000 r/min for 3 min. Fifteen milliliters of supernatant were transferred to a weighing bottle, dried at 65 °C until no water trace remained, then further dried at 105 °C for 5 h and weighed. After subtracting blank solute content from corresponding extraction solutions, solute content per milliliter was calculated (g/mL).

1.3.3 Protein Content Determination in Extraction Solution Protein content was measured using Thermo Scientific Protein Assay Kit (Product No. 23225) based on the principle that Cu^{2+} is reduced by protein under alkaline conditions to form a purple complex with absorbance at 562 nm linearly related to protein concentration. Twenty-five microliters of diluted sample and 200 L working solution were added to a microplate, sealed, shaken for 30 s, incubated at 37 °C for 30 min, cooled to room temperature, and absorbance was measured at 562 nm using a microplate reader.

1.4 Statistical Analysis

SAS 9.0 MEANS procedure was used for basic statistical analysis. PROC GLM was employed for two-way ANOVA in Experiment 1, with Duncan' s multiple comparison conducted among 12 treatments when interaction effects were significant. PROC GLM was similarly used for one-way ANOVA in Experiment 2. Duncan' s multiple comparison tested differences between treatments in both experiments. Data are presented as means ± standard deviation.

Results

2.1 Effects of Extraction Solution and Solid-Liquid Separation Method on Non-Starch Polysaccharide Enzyme Activities

Among the four extraction solutions, phosphate buffer yielded the highest xylanase activity ($P < 0.05$), while the other three solutions produced slightly lower activities. β-Glucanase activity was lowest after deionized water extraction ($P < 0.05$) and comparable among acetic acid-sodium acetate buffer, phosphate buffer, and 0.9% NaCl solution ($P > 0.05$). β-Mannanase activity was highest with deionized water extraction, followed by acetic acid-sodium acetate buffer, both significantly exceeding phosphate buffer and 0.9% NaCl solution ($P < 0.05$). Extraction solution type did not significantly affect α-galactosidase activity ($P > 0.05$).

Solid-liquid separation method had no significant effect on xylanase activity ($P > 0.05$). No separation significantly reduced β-glucanase activity ($P < 0.05$), while centrifugation or filtration significantly decreased α-galactosidase activity ($P < 0.05$). A highly significant interaction existed between extraction solution type and solid-liquid separation method for all four non-starch polysaccharide enzymes ($P < 0.01$).

For xylanase, filtration after deionized water extraction yielded significantly

higher activity than no separation or centrifugation ($P < 0.05$), while separation method did not significantly affect activity after acetic acid-sodium acetate buffer extraction ($P > 0.05$). No separation after phosphate buffer extraction produced significantly higher activity than centrifugation or filtration ($P < 0.05$), and centrifugation or filtration after 0.9% NaCl extraction yielded significantly higher activity than no separation ($P < 0.05$).

For β-glucanase, separation method did not significantly affect activity after deionized water extraction ($P > 0.05$), while centrifugation after acetic acid-sodium acetate buffer extraction produced significantly lower activity than no separation or filtration ($P < 0.05$). Centrifugation after phosphate buffer or 0.9% NaCl extraction yielded the highest activities.

For β-mannanase, no separation or centrifugation after deionized water extraction produced significantly higher activity than filtration ($P < 0.05$), while centrifugation after acetic acid-sodium acetate buffer extraction yielded significantly higher activity than no separation or filtration ($P < 0.05$). Separation method did not significantly affect activity after phosphate buffer extraction ($P > 0.05$), and filtration after 0.9% NaCl extraction produced lower activity ($P < 0.05$).

For α-galactosidase, centrifugation after deionized water, phosphate buffer, or 0.9% NaCl extraction yielded the lowest activity ($P < 0.05$), while filtration or centrifugation after acetic acid-sodium acetate buffer extraction also produced the lowest activity ($P < 0.05$).

2.2 Effects of Extraction Solution on Solubility of Non-Starch Polysaccharide Enzymes

Due to significant centrifugation effects on α-galactosidase activity in Experiment 1, solubility testing for this enzyme was not conducted in Experiment 2. Table 3 presents solute contents in centrifuged solutions of three solid enzyme preparations. For xylanase, solute content ranked as: acetic acid-sodium acetate buffer > deionized water > 0.9% NaCl solution > phosphate buffer, with significant differences between all pairs except deionized water versus 0.9% NaCl solution ($P > 0.05$). For β-glucanase, the ranking was: deionized water = 0.9% NaCl solution > phosphate buffer > acetic acid-sodium acetate buffer, with no significant difference between deionized water and 0.9% NaCl solution ($P > 0.05$) but significant differences among all other pairs ($P < 0.05$). For β-mannanase, the order was: 0.9% NaCl solution > deionized water > acetic acid-sodium acetate buffer > phosphate buffer, with no significant difference between deionized water and 0.9% NaCl solution ($P > 0.05$) but significant differences among all other treatments ($P < 0.05$).

2.3 Effects of Extraction Solution on Protein Solubility of Non-Starch Polysaccharide Enzymes

Table 4 shows protein contents in centrifuged solutions of three solid enzyme preparations. For xylanase, protein content ranked: deionized water > phosphate buffer > 0.9% NaCl solution > acetic acid-sodium acetate buffer, with significant differences among all pairs except phosphate buffer versus 0.9% NaCl solution ($P > 0.05$). For β-glucanase, the order was: deionized water > 0.9% NaCl solution > phosphate buffer > acetic acid-sodium acetate buffer, with significant differences among all treatments ($P < 0.05$). For β-mannanase, the ranking was: deionized water > 0.9% NaCl solution > phosphate buffer > acetic acid-sodium acetate buffer, with acetic acid-sodium acetate buffer significantly different from all other treatments ($P < 0.05$).

Discussion

3.1 Effects of Extraction Solution on Solubility of Solid Enzyme Preparations

Most feed enzyme products consist of enzyme proteins from microbial fermentation, adsorbed onto carriers such as wheat bran, wheat middlings, corn cob powder, and protectants, forming powder formulations. Some heat-sensitive enzymes are encapsulated in coating materials with insulating carriers to reduce activity loss during pelleting and extrusion. Carrier types and contents vary substantially among products from different manufacturers and with different activity specifications. Dissolved solutions contain soluble substances from both fermentation concentrates and carriers. Enzyme solubility characteristics depend on enzyme protein properties and environmental physicochemical factors including pH, temperature, ionic strength, and dielectric constant, potentially resulting in considerable variation among enzyme preparations from different sources.

Gao et al. reported that β-glucanase from different manufacturers requires different extraction solutions for optimal extraction. However, assay kits from Ireland' s Megazyme and national standard methods use acetic acid-sodium acetate buffer for enzyme sample extraction. In this study, acetic acid-sodium acetate buffer generally produced higher measured enzyme activities than other extraction solutions for all four enzymes. The four buffers showed substantial differences in solubility for xylanase, β-glucanase, and β-mannanase products, with different extraction solutions maximizing solubility for different enzymes. Nevertheless, acetic acid-sodium acetate buffer yielded the lowest protein content in supernatants of these three enzymes, indicating relatively low non-enzyme protein impurity levels. Therefore, acetic acid-sodium acetate buffer most effectively extracts enzyme protein compared to the other three solutions.

3.2 Effects of Solid-Liquid Separation Method on Enzyme Activity in Dissolved Solid Enzyme Preparations

Non-starch polysaccharide enzymes typically have molecular masses of 2$×10^{4}$ to 20$×10^{4}$ Da, and low centrifugal forces (e.g., 1,500×g) do not precipitate dissolved enzymes. Consequently, low-speed centrifugation is commonly used to separate dissolved enzyme solutions from insoluble residues after solid enzyme preparation dissolution. Megazyme' s non-starch polysaccharide enzyme activity assay kits employ low-speed centrifugation or filtration to obtain test enzyme solutions. Lu et al. reported that enzyme proteins may be adsorbed by filter media, with adsorption positively correlated with molecular weight, thereby reducing measured enzyme activity in filtrates.

In this experiment, no separation reduced β-glucanase and β-mannanase activities, possibly because small amounts of enzyme protein remained adsorbed on carriers and were not released, resulting in slightly lower measured activities. Both centrifugation and filtration substantially reduced α-galactosidase activity, likely because α-galactosidase was difficult to elute from carriers at the 2:100 enzyme-to-solution ratio, with most enzyme remaining on carriers during separation—a phenomenon requiring further verification. Filtration reduced β-mannanase activity, possibly due to filter paper retention of β-mannanase. Therefore, α-galactosidase extracts should not undergo solid-liquid separation, while the other three enzymes are suitable for centrifugal separation under these experimental conditions.

Conclusions

1. Acetic acid-sodium acetate buffer most effectively extracts enzyme protein from the four solid enzyme preparations, enabling optimal enzyme activity expression.
2. α-Galactosidase extracts should not be subjected to solid-liquid separation by centrifugation or filtration, whereas the other three enzyme extracts are suitable for centrifugal separation under the conditions of this experiment.

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Note: Figure translations are in progress. See original paper for figures.