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Effects of chemical modification by chitooligosaccharide on enzyme activity and stability of yeast β-D-fructofuranosidase

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ABSTRACT

The soluble chitooligosaccharides (COS) was used to modify yeast β-D-fructofuranosidase (YFF) (EC 3.2.1.26). Effects of pH, temperature, reaction time, the ratio of COS/YFF contents, and sucrose content on the modification process were studied by orthogonal experiments. The changes in enzymatic properties, kinetic parameters, infrared spectra (IR), ultraviolet absorption spectra (UAS), ultraviolet differential spectra (UDS), and fluorescence emission spectra (FES) of enzyme after modification were investigated. The activity of the modified enzyme (COSFF) was increased by 153.5% as compared to that of YFF activity. The optimal pH for COSFF was 4.5 while that for YFF was 5.0. The optimal temperature of the enzyme was unchanged after modification. The stability of COSFF was improved to certain extent. COSFF retained 98% activity after incubation at pH 4.0, 50 °C for 2 h, while YFF remained only 59.8%. Residual activity of YFF and COSFF incubated at 60 °C for 2 h was 9% and 29%, respectively. K_m of YFF was 91.11 mmol/L and v_{max} was 1.111 mmol reducing sugar/(mg min). K_m of COSFF was reduced to 47.78 mmol/L but the v_{max} of COSFF was unchanged. After modification, IR spectra of COSFF varied obviously. The two characteristic UAS peaks were red shifted by 2 nm and 3 nm, respectively.

1. Introduction

Some macromolecules (e.g. polyglycol, carboxymethyl cellulose and dextran) and small molecules (e.g. acetyl oxide, stearic acid and phthalic anhydride) can be used as the modifying agents on enzymes. The chemical modifications on enzymes can usually reduce the antigenicity and enhance the thermostability of the enzymes. At present, chemical modifications of enzymes have been mainly focused on the side chains of amino acids, which may help to find out the essential groups in enzyme active centers [1,2], or to improve the catalytic activity of enzymes [3,4]. For instance, Song et al. [5] used phthalic anhydride (PA) to modify horseradish peroxidase (HRP) and observed that the activities of PA-HRP were increased by 7.5% and 27% as compared to those of the native HRP at pH 3 and pH 10, respectively. The K_m (8.16 mmol/L) of PA-HRP was lower than that of native HRP (K_m = 12. 99 mmol/L) and the K_{cat}/K_m of PA-HRP (7. 86×10^4 L/mmol min) was greater than that (6. 70×10^4 L/mmol min) of the native HRP. Zhang et al. [6] used methoxypolyethyethylene glycol (mPEG) to modify porcine pancreas kallikrein (PPK). Their results indicated that the modified PPK displayed an increase in molecular weight, and an increased tolerance to temperature and stability. But its catalytic activity was decreased [6]. Cao et al. [7] used mPEG to modify recombinant L-asparaginase and their results indicated that the modified enzyme was more stable than the unmodified one. Thus, investigations in chemical modifications on enzymes have been carried out to reform the enzymes. However, the chemical modifications on the non-essential groups of the enzymes, for example, the carbohydrate chains of glycoproteins, have been rarely investigated.

Chitooligosaccharide (COS) is a soluble polysaccharide. In COS molecules, glucosamines or acetyl glucosamines combine each other through β-1,4-glucosidic bonds. COS is innocuous, safe and easy to be decomposed biologically. Therefore, COS has been widely used in food, medication, cosmetic, weave [8], aquiculture, agriculture [9], and other areas.

β-D-Fructofuranosidase (EC 3.2.1.26) belongs to glycoside hydrolase GH32 family. In yeast cells, there are two kinds of β-D-fructofuranosidase, i.e. the intracellular enzyme and the extracellular one. The extracellular enzyme has a higher level of glycosylation than the intracellular one does. The extracellular enzyme is the main active β-D-fructofuranosidase in yeast cells. β-D-Fructofuranosidase can hydrolyze sucrose to glucose and

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fructose, which can be used to make syrup. The sweetness of syrup is about 20% higher than that of sucrose but the crystallinity of syrup is low. And the color of syrup is shallower than that of the products of sucrose by acid hydrolysis. Thus, β-D-fructofuranosidase is widely used in foodstuff and vintage industry [10] and is also widely used in fermentation industry to produce alcohol [11], lactic acid, glycerol, and man-made honey [12]. Furthermore, β-D-fructofuranosidase can also be used in cosmetic, medicament, and paper making. However, the higher cost, lower activity in vitro, and instability of β-D-fructofuranosidase restrict its wider use in industry. Some investigators immobilized β-D-fructofuranosidase onto silicon layer [13], hydroxyethyl methacrylic acid resin [14], and nanometer hydrogel [15]. Although these practices can increase its stability, the activities of the immobilized enzymes are decreased greatly.

The purpose of this study was to promote the activity and stability of yeast β-D-fructofuranosidase (YFF) through chemical modification with COS. In this study, COS was used to chemically modify YFF. The effects of pH, temperature, reaction time, the ratio of COS and enzyme dosage, and substrate concentrations on enzyme modification were investigated. Furthermore, the variations of the conformation and the configuration of the enzyme between the modified- and unmodified-YFF were comparatively analyzed by the methods of ultraviolet absorption spectrum, ultraviolet differential spectrum and fluorescence emission spectrum. The results indicated that chemical modification of yeast YFF with appropriate COS molecules significantly improved the activity and stability of YFF through a relatively cheap and efficient way.

2. Materials and methods

2.1. Material and equipments

Yeast β-D-fructofuranosidase (YFF), 2,4,6-trinitrobenzene sulfonic acid (TNBS), bovine serum albumin (BSA), and sucrose were purchased from Sigma Company (St. Louis, MO, USA). Coomassie bright blue G-250 and Coomassie bright blue R-250 were purchased from Fluka Company. D-Fructose and D-glucose were purchased from Amresco Company. Chitooligosaccharide (COS) was offered by Shanghai Taohe Biological Technical Co (Shanghai, China). All the other chemicals were of analytical grade.

Gel Imaging System was obtained from Zhuhai Heima Co. (Zhuhai, Guangdong, China). TFD5503 Freeze-Dryer was purchased from Shin Lab Co. Ltd. (South Korea). Nicolet AVATAR 360 Fourier Infrared Spectrometer was obtained from E.S.P Co. (Eatontown, NJ, USA). UV3010 Spectrophotometer and F4500 Fluorescence Spectrometer were purchased from Hitachi Co. (Japan).

2.2. Identification of YFF purity

Polyacrylamide gel electrophoresis (PAGE) was employed to identify the purity of merchandize YFF. The concentration of spacer gel was 4.5% (w/v) and separation gel was 7.5% (w/v). The sample applied was 10 μL/well. The voltages were initially set at 150 V when the sample was running in spacer gel and changed to 200 V when the sample reached the separation gel. After electrophoresis the gel was unglued, and stained with Coomassie bright blue R-250 solution for 30 min and destained by 0.25 mol/L NaCl for 10 h [16]. The pictures of the gel were taken by using gel-imaging system.

2.3. Chemical modification of YFF with COS

2.3.1. Selection of appropriate modifiers in different molecular weight

Chitooligosaccharide (COS) with an average MW of 5000 and chitosan with an average MW of 20,000 were used to modify YFF.

A reaction system containing 25 mg YFF dissolved in 5 mL of 0.1 mol/L pH 4.0 sodium acetate buffer, 5 mL of 0.1 mol/L $NaIO_4$ and 0.5 g of sucrose was stirred for 30 min. The reaction was stopped by adding 400 μL of glycol. The mixed solution was then dialyzed against a 2 L of 0.1 mol/L sodium acetate buffer for 4 h. Dialysis was repeated 3 times.

The activated YFF solution was mixed with 0.5 g of sucrose and 40 mg of COS or chitosan, which were dissolved in 2 mL of 0.1 mol/L pH 4.0 sodium acetate buffer, and stirred for 4 h. One mL of 1 mol/L $NaBH_4$ solution was then added and stirred for another 4 h. The reaction mixture was dialyzed in a 2 L of 0.1 mol/L sodium acetate buffer for 4 h. Dialysis was repeated 3 times. Then the modified enzyme, named COSFF, was obtained.

All procedures described above were carried out at 4 °C in the dark room.

2.3.2. Screening of the optimal modification conditions

The effects of pH, temperature, reaction time, ratio of COS/YFF (25 mg), substrate concentrations on YFF modification process were investigated. Four (4) levels were set for each factor. L_{16} (4^5) orthogonal layout was used in orthogonal experiments to screen the optimal modification conditions. Variance analysis was used to check the reliability of experimental results. This confirmation experiment was repeated for 4 times.

2.4. Assays of YFF and COSFF activities

Two mL of 1 mol/L sucrose (dissolved in 0.1 mol/L sodium acetate buffer, pH 5.0) was incubated at 50 °C for 10 min. Then 0.1 mL of enzyme solution (either YFF or COSFF) was added. After 10 min, the reaction was stopped by adding 0.1 mL of 2 mol/L NaOH. The contents of reducing sugar in the solution were determined according to the method described previously [17] using glucose and fructose (1:1) as the standards.

Protein content was determined by the Bradford method [18] using bovine serum albumin (BSA) as standard.

One unit of enzyme (YFF or COSFF) activity was defined as the amount of enzyme required to produce 1 μmol reducing sugar per min under the described conditions.

2.5. Determination of saccharide content and amino modification ratio

Neutral sugar was determined by the phenol-sulfuric acid method [19] using glucose as the standard. Free amino groups of YFF and COSFF were measured by TNBS method [20]. One mL of enzyme solution was mixed with 1 mL of 4% (w/v) $NaHCO_3$ solution (pH 8.5) and 1 mL of 10% (w/v) sodium dodecyl sulfate (SDS) solution. After 20 min, 1 mL of 0.1% (w/v) TNBS was added to the mixture. The mixture was kept at 40 °C for 2 h. Then 0.5 mL of 1 mol/L HCl was added to the mixture to terminate the reaction. The absorbance of the solution was detected at 335 nm and the free amino group contents of the enzyme proteins were calculated [20]. Amino residue ratio was calculated according to the free amino group contents of enzyme solutions with the same concentration of protein before and after modification.

$$\text{Amino modification ratio}(\%) = 1 - \text{amino residue ratio}(\%)$$

2.6. Changes of YFF enzymatic properties after modification

2.6.1. Optimum pH and pH stability of YFF and COSFF

The hydrolytic activities of YFF and COSFF were measured in the 0.1 mol/L sodium acetate buffer with pH 3.0–6.0 and in 0.1 mol/L phosphate buffer solution with pH 7.0–8.0, respectively, to confirm the optimum pH.

The pH stability of enzymes was determined as follow. YFF or COSFF preparations were incubated at 50 °C in 0.1 mol/L sodium acetate buffers (pH from 3 to 5), and in 0.1 mol/L phosphate buffer solution (pH from 5.5 to 8.0), respectively. Aliquots were taken at the scheduled time points to assay the enzyme activities.

2.6.2. Optimum temperature and thermal stability of YFF and COSFF

The hydrolytic activities of YFF and COSFF were measured at different temperatures ranging from 20 °C to 80 °C in 0.1 mol/L sodium acetate buffer with pH 5.0.

To estimate the thermal stability of enzymes, YFF or COSFF preparations were incubated at 60 °C and 65 °C, respectively, in 0.1 mol/L sodium acetate buffer with pH 5.0. Aliquots were taken and quickly chilled at the scheduled time points for enzyme activity assays.

2.6.3. Assays of enzyme kinetic parameters

The catalytic velocities of YFF and COSFF were determined with different concentrations of sucrose. The V_{max} and K_m of YFF and COSFF were calculated by Lineweaver–Burk curves.

2.7. Spectrum analysis

The infrared spectra, the ultraviolet absorption spectra and the fluorescence emission spectra of YFF and COSFF were measured.

The pellets for Fourier transform-infrared (FT-IR) spectroscopy analysis were prepared by mixing the freeze-dried enzymes with KBr. FT-IR spectra measurements were performed with a Nicolet AVATAR 360 spectrometer with DTGS TEC detector, from 400–4000 cm^{-1}.

The ultraviolet absorption spectra of YFF and COSFF and the ultraviolet differential spectrum of COSFF were measured on UV3010 Spectrophotometer at the wavelengths of 190–400 nm.

The fluorescent 3-D spectra of YFF and COSFF were measured on F4500 Fluorescent Spectrometer with 0.1 mol/L pH 5.0 sodium acetate buffer as the control solution to confirm the excitation wavelength. The fluorescent emission spectra of YFF and COSFF were then measured and compared each other.