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New insights into heat induced structural changes of pectin methylesterase on fluorescence spectroscopy and molecular modeling basis

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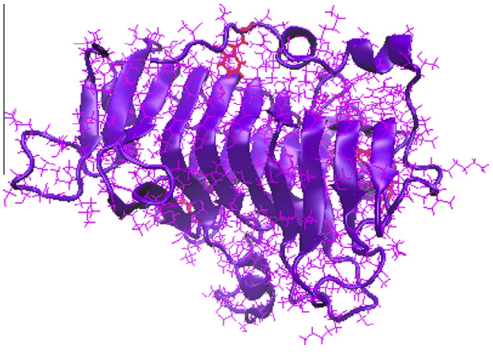
HIGHLIGHTS

- Spectroscopy results indicate different molecular species at high temperatures.
- Molecular modeling suggested significant conformational changes.
- The changes in PME structure over 55 °C lead to enzyme inactivation.
- Good correlation between the inactivation and fluorescence results was suggested.

GRAPHICAL ABSTRACT

3D structure of PME. The molecular motifs are represented in New Cartoon style. The Trp residues are represented in Bonds style.



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ABSTRACT

Heat-induced structural changes of *Aspergillus oryzae* pectin methylesterase (PME) were studied by means of fluorescence spectroscopy and molecular modeling, whereas the functional enzyme stability was monitored by inactivation studies. The fluorescence spectroscopy experiments were performed at two pH value (4.5 and 7.0). At both pH values, the phase diagrams were linear, indicating the presence of two molecular species induced by thermal treatment. A red shift of 7 nm was observed at neutral pH by increasing temperature up to 60 °C, followed by a blue shift of 4 nm at 70 °C, suggesting significant conformational rearrangements. The quenching experiments using acrylamide and iodide demonstrate a more flexible conformation of enzyme with increasing temperature, especially at neutral pH. The experimental results were complemented with atomic level observations on PME model behavior after performing molecular dynamics simulations at different temperatures. The inactivation kinetics of PME in buffer solutions was fitted using a first-order kinetics model, resulting in activation energy of 241.4 ± 7.51 kJ mol^{-1}.

Introduction

Pectin, one of the most complex macromolecules present in the plant cell wall, greatly influences the textural properties of the plant cell wall. Therefore, pectin and the related textural changes

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are widely studied in plant-based food processing. Pectin can undergo a variety of chemical or enzymatic conversions, modifying its structural and functional properties. Physicochemical transformations of pectins induced as a consequence of the hydrolytic activity of pectic enzymes, such as polygalacturonase and pectin methylesterase (PME), cause changes of the sensory attributes and technological properties of fruit and vegetable products, during maturation, storing and canning.

PME (EC 3.1.1.11) catalyzes the hydrolysis of methyl ester groups from the pectin (a polymer of γ-1.4 linked galacturonic acid and galacturonic acid methylester). The enzyme is involved in the first step of the fruit-ripening process, by producing pectin with a lower degree of methylation which, in turn, becomes the substrate of polygalacturonase [1]. In absence of polygalacturonase activity, pectate chains can be cross-linked via calcium bridges and form intermolecular networks [2]. In addition, pectin with a lower degree of esterification is less sensitive to non-enzymatic depolymerization (β-elimination) that can occur during heating [3].

PME is used for various applications in fruit processing e.g. texture improvement of fruit pieces [4], juice extraction, concentration and clarification of fruit juices [5]. PME is therefore one of the key enzymes in fruit and vegetable processing. Endogenously present plant PME can positively or negatively affect structural quality of plant-based foods (cloud stability, viscosity, and texture). On the other hand, PME, together with other pectinases, finds (actual or potential) applications as an exogenous processing aid. Food processing can be applied either to inactivate or to boost the enzymatic activity.

Most of the exogenous PME used in food processing is obtained from fungal sources, mainly *Aspergillus* species. From a structural point of view, PMEs show some interesting structural features, the most prominent being the presence of β-helices [6]. Technological applications of PMEs from different fruits and vegetables have been investigates as follows: apples [7], banana [8], kiwi and kaki fruits [9], potatoes and carrots [10,11], tomatoes [12], etc.

Traditionally, thermal processing is the most commonly applied preservation technology since it allows efficient inactivation of both pathogenic and spoilage microorganisms and quality-related enzymes. Thermal stability of PME is well documented, mainly from inactivation kinetic studies.

However, little is known about structural particularities of PME at different temperatures. The structure and heat-induced dynamics of protein molecules in solution can be extensively investigated by combining fluorescence methods due to the sensitivity of intrinsic fluorescence of tryptophan residues to their microenvironments and molecular modeling approach [13].

In the present work we have studied the emissive properties of commercial PME from *Aspergillus oryzae* PME, focusing on structural and conformational changes at different temperature values ranging from 25 °C to 70 °C. The fluorescence spectroscopic measurements were employed to characterize the heat-induced changes in enzyme conformation at pH 4.5 and 7.0. Thus, changes in intrinsic fluorescence parameters (intensity of fluorescence, maximum position, anisotropy, and parameter *A* value), "phase diagram" method and quenching experiments using acrylamide and iodide were applied. For a more comprehensive characterization of heat induced structural changes of PME, molecular dynamics simulations were carried out and the results were further correlated with the inactivation kinetics parameters.

Experimental

Materials

Commercial PME from *A. oryzae* (NovoShape®) was purchased from Novozymes (Denmark). The enzyme product was used without further purification. Apple pectin (esterification degree of 75%) was obtained from Fluka Chemical Co. (Switzerland). Acrylamide was purchased from Sigma (Sigma–Aldrich Co., St. Louis, MO). All other chemicals used were of analytical grade.

The samples were obtained by diluting the commercial solution (1:20) with 0.01 M phosphate buffer (pH 4.5) and 0.01 M Tris buffer (pH 7.0). The protein concentration in the samples was determined using Sigma Procedure No. TPRO-562 (for kit No. BCA-1 and Product No. B-9643).

Heat treatment

The enzyme heat treatment was performed in different conditions, depending on the investigation technique used.

For fluorescence spectroscopy studies, the protein solutions (0.050 mL) were filled in plastic tubes (1 cm diameter). The thermal treatment experiments were conducted in a thermostatic water bath at temperatures ranging from 25 to 70 °C for 10 min. The total time of the thermal treatment was long enough to ensure the structural rearrangements within the protein molecule, taking into account the low protein concentration used in the experiment. After the thermal treatment, the tubes were immediately cooled in ice water to prevent further denaturation. All measurements were performed within 2 min after the heat treatment. All spectroscopic studies were carried out on the heated–cooled protein, implying that only irreversible/permanent structural changes were detected. Prior to each experiment, 50 μL of protein solution were suspended in 3 mL buffer of pH 4.5 or 7.0 and allowed to stand at room temperature for 15–30 min.

Thermal inactivation experiments of PME at pH 4.5 were conducted using the test tube method. Aliquots (0.100 mL) of protein solutions were placed in test tubes and immersed in a water bath (Digibath-2 BAD 4, RaypaTrade, Spain) at temperatures ranging between 50 °C and 65 °C for different holding times (0–10 min). After thermal treatment, the test tubes were immediately immersed in ice water to allow rapid cooling.

Fluorescence spectroscopy experiments

All fluorescence spectroscopy experiments were performed on a LS-55 luminescence spectrometer (PerkinElmer Life Sciences, Shelton, CT) with a quartz cell of 0.1 M path length.

Phase diagram method

Partially folded conformations of proteins can be distinguished by using spectroscopic data in a form of spectral diagrams. The phase diagram method is based on Eq. (1):

$$I(\lambda_1) = a + bI(\lambda_2) \quad (1)$$

where $I(\lambda_1)$ and $I(\lambda_2)$ are the spectral intensity values measured on wavelengths λ_1 and λ_2 under different experimental conditions for a protein undergoing structural transformations; a and b are the intercept and respectively the slope of the $I(\lambda_1)$ versus $I(\lambda_2)$ plot.

Intrinsic fluorescence

Intrinsic fluorescence measurements consist in excitation at λ_{ex} 295 nm. The emission spectrum was collected between 300 and 420 nm. The excitation and emission slits were both 10 nm, and the scan speed was 500 nm min^{-1}. Measurements were made at 25 °C.

The position and form of the fluorescence spectra were characterized also in terms of parameter A $(I_{320}/I_{365})_{292}$, where I_{320} and I_{365} are fluorescence intensities at λ_{em} of 320 and 365 nm, respectively, for λ_{ex} of 292 nm [14].