Isolation and purification of an alkaline esterase from *Sparassis crispa*

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Introduction

Esterases (EC.3.1.1.1) and lipases (EC.3.1.1.3) catalyze the hydrolysis and synthesis of ester compounds. Currently, an increasing number of drugs and organic materials have been produced through esterase- and lipase-catalyzed reactions. Esterase hydrolyzes esters of short-chain carboxylic acids while lipase shows hydrolytic activity toward insoluble long-chain acylglycerides [1]. Hence, these enzymes differ in substrate specificity and type of enzyme kinetics. In this regard, the use of target-specific enzymes for the efficient production of such target materials is essential. These enzymes are found in animals, plants and microorganisms [2]. In view of the biocatalytic applications, it is of great interest to study the specificity and selectivity of novel esterase. Potential applications of these enzymes in the industry cover a broad spectrum, including additives, in food processing, in environmental bioremediation, and complete degradation of plant materials for the production of useful organic compounds [3,4]. Mushrooms have been used for centuries in Korea both as food and medicine. *Sparassis crispa* is also known as cauliflower mushrooms. It is an edible/medicinal mushroom. For the present study, we have taken the fruiting body of the mushroom. Fruiting body of this mushroom is light brown yellow to yellow grey or a creamy white cauliflower color. They are normally 10 to 25 cm tall; fruiting bodies are more than half a meter tall. The cultivated mushrooms contain little fat and digestible carbohydrates, but they have higher protein and glucan contents than most vegetables. Apart from this, they are also rich in vitamins B, D, K, A and C, which is suitable for low calorie diets [5,6,7,8]. It is reported that this mushroom has significant antioxidant and free radical scavenging activities [9]. In addition, mushrooms are good enzyme sources. In particular esterases are valuable enzymes because of their novel characters which found to have many applications in industry. The present study reports the purification and biochemical properties of an alkaline esterase from *S. crispa*. The characterization of an esterolytic enzyme from *S. crispa* was studied in terms of substrate specificities, thermostability, pH stability and pH optimum.

Experimental

Sample collection and extraction

S. crispa mushroom samples were collected from South Korea and Japan. The fresh fruiting body of the mushroom was freeze dried. Crude enzyme extracts were prepared as reported previously with some modification [10].The frozen fresh fruiting body (50 g) of mushroom was ground to a fine powder in liquid nitrogen using prechilled ceramic mortar pestle. Then, the mushroom sample was separately extracted in acetate buffer pH 5.0, phosphate buffer pH 7.0 and Tris-HCl buffer pH 9.0 containing 2 mM EDTA, 1 mM $MgCl_2$ and 1 mM phenylmethylsulfonyl fluoride (PMSF) at 4°C. All the buffers were cold and at 10 mM concentration. The suspension was centrifuged at 10,000 rpm for 10 min at 4°C. After that supernatant was used as crude enzyme extracts.

Enzyme purification

The enzyme was isolated from the crude sample by using preparative isoelectric focusing cell (BioRad, USA) and gel filtration chromatography. The crude enzyme was concentrated by ammonium

sulphate precipitation at 85% saturation. After that the solution was centrifuged at 8,000 rpm for 10 min. The precipitate was redissolved in 10 mM Tris-HCl buffer pH 9.0 and dialyzed overnight against the same buffer. The concentrated esterase enzyme was subjected to isoelectric focusing to determine the pI of the protein. The fractions was further purified by Sephadex G 75 column, which is equilibrated with 10 mM Tris –HCl buffer pH 9.0 containing 0.15 M NaCl and was eluted with the same buffer with a flow rate of 0.5 ml/min. Purification was monitored by activity assays and SDS-PAGE. All protein purification steps were performed at 4° C.

Enzyme assay

Esterase activity was assayed by measuring the amount of *p*-nitrophenol (*p*NP) released [11]. Activity was measured using a *p*-nitrophenyl butyrate (*p*NPB) or other *p*-nitrophenyl esters (*p*NPEs) as substrates. The substrate mixture was prepared by mixing *p*NPB solution, ethanol and 10 mM Tris – HCl buffer pH 9.0 in the ratio of 1:4:95 (v/v/v), respectively. For the enzyme reaction mixture, 0.6 ml of the crude enzyme extract was added to 1.8 ml of the substrate solution. The enzyme substrate mixture was incubated at 30°C for 20 min. The reaction was monitored spectrophotometrically at 405 nm for the release of *p*NP. The esterolytic activity was determined by the amount of *p*NP liberated by *p*NP standard graph. The non enzymatic reaction was done by using a blank without enzyme. One unit of enzyme activity was defined as the amount of enzyme needed to release 1μmol of *p*-nitrophenol per min under assay conditions.

Protein analysis

Protein quantification is done by BCA method using BSA as standard [12]. 10% SDS-polyacrylamide gel electrophoresis was performed by the Laemmli method [13].

Results and discussion

In the present study, esterase activity of *S. crispa* mushroom was evaluated. The considerable esterase activity was observed in the crude extract by using *p*-nitrophenyl butyrate as the substrate. In all our studies, the *S. crispa* enzyme extract prepared with 10 mM Tris-HCl buffer, pH 9.0 was taken for further study because of the highest estrolytic activity. A typical procedure for the purification of the esterase is summarized in Table1.

SDS-PAGE results showed the molecular weight of 65 kDa (Fig. 1) and a zymogram analysis revealed that the purified esterase could hydrolyze tributyrin (TBN) substrate (Fig. 1). The isoelectric point of this protein is 9.5. The optimum enzyme activity occurred at pH 9.0, thus indicating an alkaline esterase (Fig. 2). The isolated enzyme showed the purity about 83 fold with the recovery of 0.98%. Substrate specificity of the crude enzyme was examined towards several *p*-nitrophenyl esters having different chain lengths. The highest activity was obtained in *p*-nitrophenyl butyrate, but no activity or very slight activity was obtained in *p*-nitrophenylpalmitate (Fig. 3). This result revealed that the enzyme is esterase not a lipase. Esterases are known to hydrolyze short-chain substrates whereas lipases are known to hydrolyze long chain substrates. This confirms that the enzyme is an esterase rather than a lipase [14].

Table 1. Purification step of esterase from *S. crispa*

Fraction	Total protein (mg)	Total activity (U)	Sp. activity (U/mg)	Yield (%)	Purification (fold)
Crude extract	841.5	2037.5	2.4	100	1
Ammonium sulfate ppt	161.6	1034.75	6.4	50	2.6
Preparative IEF	0.83	94	130.74	4.6	54.4
Gel filtration	0.1	28	200	0.98	83

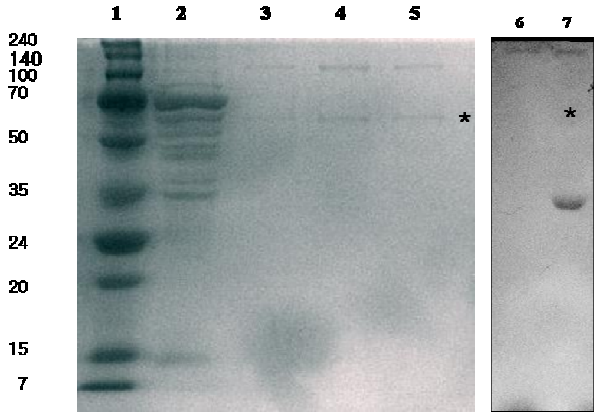

Figure.1 SDS-PAGE of esterase Lane: 1, marker proteins; 2, $(NH_4)_2SO_4$-precipitated proteins; 3-5 were the purified esterase; 6, an asterisk mark indicates the target protein and TBN hydrolysis-zymogram.

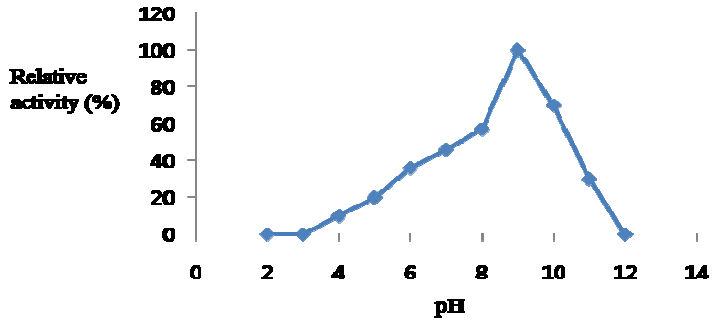

Figure 2. Effects of pH on the activity of esterase. The activity was measured at various pH values and presented as a percentage of the maximum activity, taken as 100%.

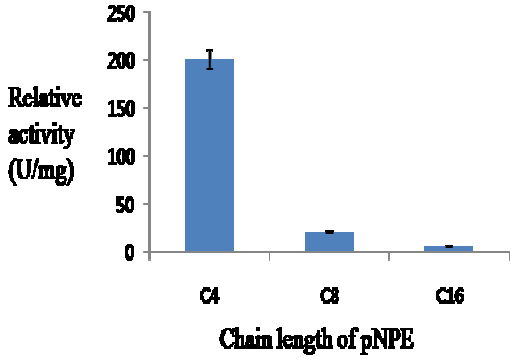

Figure 3. Substrate specificity of the esterase for *p*-nitrophenyl esterase. The *p*-nitrophenyl released from the substrates was measured. C4: *p*-nitrophenyl butyrate; C8: *p*-nitrophenylcaprylate; C16: *p*-nitrophenylpalmitate.

Conclusion

For the present study, we have taken the fresh fruiting body of the mushroom. The fruiting bodies of *S. crispa* have been reported to exhibit a significant effect to cure gastric ulcer and esophageal cancer. Fruiting body of this medicinal mushroom *S. crispa* produces an esterase with a high molecular weight, a high thermostable and alkaline property. The alkaline nature of this enzyme is helpful for industrial applications. The present study gives information for investigating new mushroom materials for food industry. In addition, this new alkaline esterase could potentially be used as a biocatalyst in hydrolysis and synthesis reactions performed within the pharmaceutical and fine chemical industries.

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