Separation and Purification Technology 132 (2014) 174–181

Contents lists available at ScienceDirect

Separation and Purification Technology

journal homepage: www.elsevier.com/locate/seppur

Three-phase partitioning and proteins hydrolysis patterns of alkaline proteases derived from fish viscera

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ARTICLE INFO

Article history:
Received 12 August 2013
Received in revised form 3 May 2014
Accepted 5 May 2014
Available online 21 May 2014

Keywords:
Alkaline proteases
Fish viscera
Protein hydrolysates
Three-phase partitioning
Trypsin

ABSTRACT

In this study, the recovery alkaline proteases from farmed giant catfish viscera were isolated by using three-phase partitioning (TPP). Factors affecting partitioning efficiency such as salts, solvent types, pH, and incubation temperatures were studied. Furthermore, the application of extracted alkaline proteases on proteins hydrolysis was also determined. The system consisted of crude enzyme extract:*t*-butanol 1:0.5 (w/v), 50% sodium citrate, pH 8.0 with incubation temperature of 25 °C provided the highest enzyme recovery (220%). The smear protein bands with molecular weight of 20, 24, and 215 kDa of TPP fractions appeared on the protein stained gel. Two major clear zones (24 and 130 kDa) in the interphase were observed on casein-substrate gel electrophoresis. Extracted alkaline proteases showed relatively high effective in protein hydrolysis. As a result, TPP provided high enzyme recovery and could be applied to other enzymes. The obtained alkaline proteases can be further applied in preparation of protein hydrolysates.

1. Introduction

In recent years, the trend has been developed rapid, efficient, economical and scalable approach for separation and purification of enzymes [1,2]. Three-phase partitioning (TPP), a one-step enzyme purification approach, is carried out by mixing salts i.e. ammonium sulfate, potassium phosphate, and sodium citrate and organic solvents i.e. *t*-butanol, 2-butanol, 1-propanol, and 2-propanol to obtain organic phase, interfacial precipitate and aqueous phase [3]. TPP employs collective operation of principles involved in numerous techniques like salting out, isoionic precipitation, cosolvent precipitation, osmolytic, and kosmotropic precipitation of proteins [1]. Moreover, the addition of organic solvent in the presence of salt pushes the protein out of the solution to form an interfacial precipitate layer between the lower aqueous and upper organic layers. TPP is a concentrating or dewatering step and some enzymes have enhanced catalytic activities in these conditions within short periods of time (about 1 h) [4]. TPP has been widely used to separate and purify various enzymes such as α-galactosidase from *Aspergillus oryzae* [4], protease/amylase inhibitor from wheat germ [5] and ragi (*Eleusine coracana*) [6], phospholipase D from *Dacus carota* [7], α-galactosidase from tomato [8], and pepino (*Solanum muricatum*) [9], invertase from Baker's yeast [10], and tomato [11], proteases from papaya peels [12], *Calotropis procera* latex [13] and giant catfish viscera [14], inulinase from *Aspergillus niger* [15]. Rawdkuen et al. [14] showed the best condition for separating of alkaline proteases from farmed giant catfish viscera that was consisted of the crude enzyme extract to *t*-butanol ratio of 1.0:0.5 in the presence of 50% $(NH_4)_2SO_4$. Besides, TPP was used to isolate trypsin inhibitors from navy bean, red kidney bean and adzuki bean by mixing the crude extract with solid ammonium sulfate (30% saturation, w/v) and *t*-butanol with the ratio of 1:1 (v/v) [16].

With an increase in fish processing, a large amount of internal organs and by-products will be generated. The viscera in some types of fish account for approximately 5–10% of the entire weight of the fish; the viscera percentage tends to increase with the fish body weight [14]. The most important proteases in the viscera of fish and aquatic invertebrates are aspartic protease (pepsin) and serine proteases (trypsin, chymotrypsin, collagenase, and elastase) [17]. Proteases are by far the most studied enzymes for industrial bio-processing. Almost half of all industrial enzymes are proteases, mostly used in the detergent, leather, and food industries [18]. Recently, the use of alkaline proteases from marine digestive organs, especially trypsin, has increased remarkably since they are both stable and active under harsh conditions, such as at temperatures of 50–60 °C, high pHs, and in the presence of surfactants

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http://dx.doi.org/10.1016/j.seppur.2014.05.006
1383-5866/

or oxidizing agents [18]. The enzymes recovered from fish viscera have also been successfully used as seafood processing aids including the acceleration of fish sauce fermentation [19], extraction of carotenoprotein [20], and production of protein hydrolysates [21–25]. Alkaline proteases and trypsin have been used to produce protein hydrolysates and the obtained hydrolysates showed bioactive properties such as antioxidant and angiotensin I converting enzyme (ACE) inhibitory properties [21–23,25]. Crude enzyme extract from sardine (*Sardina pilchardus*) viscera was used to produce protein hydrolysates from heads and viscera of sardinelle compared with Alcalase and provided the potent ACE inhibitory peptides with IC_{50} 1.2 mg/mL [25]. Alkaline proteases from pyloric caeca extract of three fish species including brownstripe red snapper (*Lutjanus vitta*), bigeye snapper (*Priacanthus tayenus*) and threadfin bream (*Nemipterus marginatus*) have been used to produce gelatin hydrolysates from the skin of brownstripe red snapper possessing antioxidative activities [22]. Tuna frame protein and salmon by-products were hydrolyzed by using trypsin compared to other proteases like Alcalase, Neutrase, Flavourzyme, Protamex, pepsin, papain, and α-chymotrypsin and providing antihypertensive effect [26,27].

The effect of different salts, organic solvents, pHs, and incubation temperatures on TPP has never been prevalently investigated. Previous reports indicate that significant selectivity in precipitation can be obtained by varying the concentration of ammonium sulfate and the aqueous phase-to-*t*-butanol ratio [14]. Therefore, the present work was involved in amelioration of alkaline proteases recovery from farmed giant catfish viscera and different parameters required were optimized, and finally the enzyme was used for protein hydrolysis in comparison with commercial bovine trypsin against egg white, whey protein concentrates, and soy protein isolates.

2. Materials and methods

2.1. Chemicals and raw materials

2-Methyl-2-propanol (*t*-butanol; $C_4H_{10}O$), 1-butanol (*n*-butanol; $C_4H_{10}O$), 1-propanol (*n*-propanol; C_3H_8O), 2-propanol (isopropanol; C_3H_8O) were purchased from Panreac (Barcelona, Spain). Ammonium sulfate ($(NH_4)_2SO_4$), potassium phosphate (K_2HPO_4), sodium citrate ($C_6H_5Na_3O_7{\cdot}2H_2O$) were purchased from Univar (Ajax Finechem, Australia). Trypsin from bovine pancreas (CAS No. 9002-07-07, 3312.2 U/mg), trichloroacetic acid (TCA), hydrochloric acid, sodium hydroxide, tris-(hydroxymethyl)-aminomethane and other chemicals with analytical grade were obtained from Merck (Darmstadt, Germany). Sodium dodecyl sulfate (SDS), bovine serum albumin (BSA), and casein were obtained from Fluka (Buchs, Switzerland). Beta-mercaptoethanol (β-ME) and Coomassie Brilliant Blue G-250 were purchased from Sigma Chemical Co. (St. Louis, MO, USA).

2.2. Crude enzyme extract preparation

Viscera of farmed giant catfish were obtained from Charun Farm, Chiang Rai, Thailand. Pooled viscera were immediately frozen and stored at −20 °C until used. The frozen viscera were thawed using running tap water (26–28 °C) until the core temperature reached (−2 ± 2 °C). The sample was cut into small pieces and homogenized for 2 min with extraction buffer (10 mM Tris–HCl pH 8.0, containing 10 mM $CaCl_2$) in the ratio of 1:5 (w/v). The mixture was centrifuged at 10,000*g* for 10 min at 4 °C. The pellet was discarded and the supernatant was collected and referred to as "crude enzyme extract" (CE). Protein content and protease activity in CE were measured.

2.3. Alkaline proteases partitioning

2.3.1. Effect of salts on proteases partitioning

In this study used the optimum condition according to Rawdkuen et al. [14] that using crude enzyme extract to *t*-butanol in ratio of 1.0:0.5 in the presence salt of 50% (w/v). The effect of salts on alkaline proteases partitioning was carried out by using different salts; $(NH_4)_2SO_4$, K_2HPO_4, and $Na_3C_6H_5O_7$ at the concentration of 50% (w/v). The salts were added to CE in 50 mL centrifuge tubes. The solutions in the tubes were mixed vigorously to dissolve the salt for 5 min, followed by adding *t*-butanol in the ratio of 1:0.5 (v/v) (ratio of crude extract to *t*-butanol). The mixture was vortexed for 1 min, and shaken at 90 rpm at ambient temperature (27–33 °C) for 60 min. Then, the mixtures were then centrifuged at 5000*g* for 10 min at 4 °C. Three-phase formed and were collected separately. The lower aqueous layer was collected by piercing and the organic phase (upper phase) was removed carefully with a Pasteur pipette. All fractions were measured for volume and recorded. Afterward, the interfacial precipitate containing alkaline proteases was dissolved in 10 mM Tris–HCl buffer (pH 8.0) containing 1 mM $CaCl_2$ by the ratio 1:1 (w/v) and dialyzed in distilled water for 12 h at 4 °C. A change in distilled water at 3 h intervals was required for efficient removal of salt and *t*-butanol bound to the protein aggregates. The dialyzed interfacial precipitate and lower aqueous phases were measured for volume, analyzed for alkaline proteases activity, and protein content. The best condition which resulted in maximum recovery was chosen for further study. All the experiments were performed in triplicates.

2.3.2. Effect of solvents on proteases partitioning

The effect of solvent types on alkaline proteases partitioning was studied by adding various organic solvents (*t*-butanol, 1-butanol, 1-propanol and 2-propanol) in the ratio of 1.0:0.5 (v/v) (ratio of crude enzyme extract to organic solvents) to the condition that provided the maximum proteases recovery from previous step. After that, collecting each phase and investigating protein content and protease activity as mentioned above. The best condition which resulted in the highest recovery was chosen for further study.

2.3.3. Effect of pHs on proteases partitioning

The effect of pHs on alkaline proteases partitioning was also investigated. After mixing crude enzyme extract and selected salt from previous step, the pH of the system was adjusted to each pH values (6, 7, 8, 9, and 10) by addition of 2 M HCl or NaOH. Next, the selected organic solvent was added to the mixture with the ratios of 1:0.5 (v/v). Partitioning for this step were done and determined as previously described. After that, collecting each phase and investigating protein content and protease activity as mentioned before. The optimum pH value providing the highest recovery was chosen for further study.

2.3.4. Effect of incubation temperatures on proteases partitioning

The best condition that provided the highest recovery from previous step was used to study the effect of incubation temperatures. The effect of temperatures on alkaline proteases partitioning was investigated by incubating the mixtures in an incubator shaker at 4, 25, 37, and 45 °C at 90 rpm for 1 h. Then, the mixtures were centrifuged at 5000*g* for 10 min at 4 °C. Partitioning for this step was done and determined as previous described. After that, collecting each phase and investigating protein content and protease activity as mentioned before. The optimum temperature providing the highest recovery was chosen for further investigation.