Process Biochemistry 46 (2011) 801–805

Contents lists available at ScienceDirect

Process Biochemistry

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

Short communication

Purification of chymotrypsin from pancreas homogenate by adsorption onto non-soluble alginate beads

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ARTICLE INFO

Article history:
Received 29 March 2010
Received in revised form
17 November 2010
Accepted 17 November 2010

Keywords:
Adsorption
Alginate beads
Chymotrypsin

ABSTRACT

Chymotrypsin was purified from an activated homogenate of bovine pancreas by adsorption onto non-soluble alginate beads in 25 mM Tris–acetate–5 mM $CaCl_2$ buffer at different adsorbate–adsorbent ratios and the pH values were assayed. Under all the experimental conditions, the enzyme has a positive net electrical charge whereas alginate is negatively charged. After performing steps of washing and desorption in 25 mM Tris–acetate–500 mM $CaCl_2$ buffer pH 7.0, chymotrypsin was purified 9 times with an enzyme recovery of 62%. The eluate fractions resulted in two bands in sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE). The method allows purification with suitable values from a raw sample like pancreas homogenate without a previous clarification step.

1. Introduction

The development of techniques and methods for the separation and purification of biological macromolecules has been an important requirement for much advancement in Biotechnology. It is often argued that the downstream processing is a critical limiting factor in the commercial development of biotechnology [1]. Most purification technologies employ precipitation of proteins as one of the initial operations aimed at concentrating the product stream for further downstream steps. Diluted feedstock often requires an additional concentration step by ultrafiltration. An initial adsorption step can also be employed for product concentration; an ion-exchange process is usually used, which has the advantage of simultaneously yielding partial purification. Thus, this adsorption can be applied for the primary recovery of a desired product directly from crude feedstock containing particulate materials such as cells, cell debris and other fine particles. This, in turn, greatly simplifies the subsequent operations in a downstream bioprocess [2].

Alginate is a natural non-toxic polysaccharide and it forms insoluble gel beads due to its ion binding properties to multivalent cations such as calcium. Alginate beads were used not only as adsorbent for protein purification but also in studies of encapsulation and delivery of biomolecules [3] and entrapment of enzymes for immobilization [4]. Sometimes, the matrix of adsorption has a specific affinity towards the protein/enzyme targeted for the separation. Alginate beads were used to isolate wheat germ alpha amylase and were purified 45 times and 70% recovery in alginate matrix, which has an inherent affinity for the enzyme [5].

The main alkaline enzymes in pancreas are chymotrypsin, trypsin and elastase, all belonging to the serine-protease family. They are characterized by serine, histidine and aspartic residues in their active site. As a group, serine proteases are active at neutral and alkaline pH, and inactive or unstable in acid pH [6]. Serine proteases are of considerable interest, in view of their activity and stability at alkaline pH and they are used in various industrial market sectors, such as detergent, food, pharmaceutical and leather. With regards to serine protease purification, researchers obtained an enzyme with chymotryptic activity from camel pancreas, which was purified 52-fold in a 48% yield by a three-step chromatographic procedure consisting of anion-exchange, cation-exchange and affinity chromatography [7]. Burton and Lowe had designed ligands, which were attached to Sepharose CL-4B and purified pancreatic kallikrein 110-fold from a crude pancreatic extract [8]. Other researchers purified ChTRP from viscera of sardine with a purification factor of 13 and 6% recovery after the saline precipitation, gel filtration and ion exchange chromatography [9]. However, these methodologies are not suitable for scaling up in industry. Boeris et al. have developed a method for the isolation and purification of chymotrypsin by precipitation with acidic polyelectrolyte from pancreas homogenate [10].

Abbreviations: ChTRP, chymotrypsin; TRP, trypsin; CAB, calcium alginate beads; BTEE, benzoyl-L-tyrosine-ethyl-ester; BAPNA, Nα-benzoyl-DL-arginine-*p*-nitroanilide.

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doi:10.1016/j.procbio.2010.11.011

The aim of this work was to assay the adsorptive behavior of non-soluble calcium alginate beads (CAB) applied to the recovery and purification of bovine chymotrypsin from its natural source. Desorption of enzymes was carried out applying high ionic strength, and the recovery and the purification factor of the process were evaluated.

2. Materials and methods

2.1. Chemicals

Alginate, bovine serum albumin, chymotrypsin (ChTRP), benzoyl-L-tyrosine-ethyl-ester (BTEE), Nα-benzoyl-DL-arginine-*p*-nitroanilide (BAPNA), sodium dodecyl sulphate (SDS) and molecular weight markers (prepared with aprotinin (6500), α-lactalbumin (14,200), trypsinogen (24,000) carbonic anhydrase (29,000), glyceraldehyde-3-phosphate dehydrogenase (36,000), ovalbumin (45,000) and bovine albumin (66,000)) were purchased from Sigma, and used without further purification. The pancreas was kindly supplied by a local slaughterhouse. All the other reagents were of analytical grade.

2.2. Alginate beads formation

Non-soluble calcium alginate beads (CAB) were prepared by dripping 3% (w/v) sodium alginate solution into a cross-linking solution composed of 0.1 M $CaCl_2$. The beads were incubated in the cross-linking solution overnight and then washed several times with distilled water [11].

2.3. Bovine pancreas homogenate preparation and activation

The pancreas was removed from a recently killed bovine, washed with isotonical saline solution and cut in small pieces, mixed with Tris–HCl–sodium citrate buffer, pH 2.5 (ratio 1:3) and homogenized for 5 min in a Waring laboratory variable speed blender at a rate of 4000 rpm thermostatized in an water-ice bath. The resulting homogenate was divided in aliquots and frozen at −30 °C without further treatment. Chymotrypsinogen and trypsinogen are inactive precursors of chymotrypsin (ChTRP) and trypsin (TRP), respectively; therefore, a previous activation step was required. The zymogen activation was initiated by adding a small aliquot of TRP (0.0001%, w/w) in 90 mM Tris–HCl–45 mM $CaCl_2$ buffer pH 8.2. The time required to complete the activation process was also determined by measuring the ChTRP and TRP activity at different intervals of time until a maximal value was reached [10].

2.4. Determination of ChTRP and TRP activity

ChTRP assay is based on the hydrolysis of BTEE [12]. The reaction rate was determined by hydrolysis of the substrate at 0.6 mM concentration in 100 mM Tris–HCl–100 mM $CaCl_2$ buffer pH 8.2. The production of benzoyl-tyrosine was measured by monitoring the increase in Abs 256 nm every 2 s for 3 min. One ChTRP unit is defined as 1 μmol of substrate hydrolyzed per minute of reaction and was calculated with the following equation: U (μmol/min) = (ΔA 256 nm/min) × 1000 × 1/964, where 964 is the benzoyl-tyrosine molar extinction coefficient [13].

TRP activity was determined with the substrate BAPNA using a method modified by Gildberg and Overbo [14]. BAPNA was used in the assay at a final concentration of 0.85 mM in 100 mM Tris–HCl buffer pH 8.2. The reaction rate was determined by measuring the absorbance of the released reaction product, *p*-nitroanilide, which absorbs at 400 nm. BAPNA hydrolysis units (U) were calculated with the following equation: U (μmol/min) = (ΔA 400 nm/min) × 1000 × 1/8800, where 8800 is the *p*-nitroaniline molar extinction coefficient [9].

2.5. Protein determination

Protein determination was carried out according to the method of Lowry et al., using bovine serum albumin as a standard.

2.6. Batch ChTRP adsorption on CAB

For adsorption of commercial ChTRP, 2 mL of 0.05% (w/w) ChTRP in 25 mM acetate–Tris–$CaCl_2$ buffer were mixed with different dry masses of CAB. Three pH values, 3.8, 5.0 and 7.0; two temperatures, 6 and 20 °C and the following ionic strengths, $CaCl_2$ 5 mM, 50 mM and 100 mM were assayed. The mixtures were stirred until the adsorption equilibrium was reached. ChTRP activity was determined over time.

For the bovine pancreas homogenate adsorption experiment, aliquots of 5–10 mL of homogenate were mixed with two different dry masses of CAB. Ratios of 2.6:1 and 0.7:1 for ChTRP units:CAB dry mass weight values were selected. The adsorption was carried out in the following conditions: 25 mM acetate–Tris–5 mM $CaCl_2$ buffer. The same temperatures and pH values were assayed. ChTRP and TRP activity and total protein were determined over time. Analyses of adsorbent-free controls over the time scale of the experiment were carried out to evaluate the stability of the ChTRP in that period.

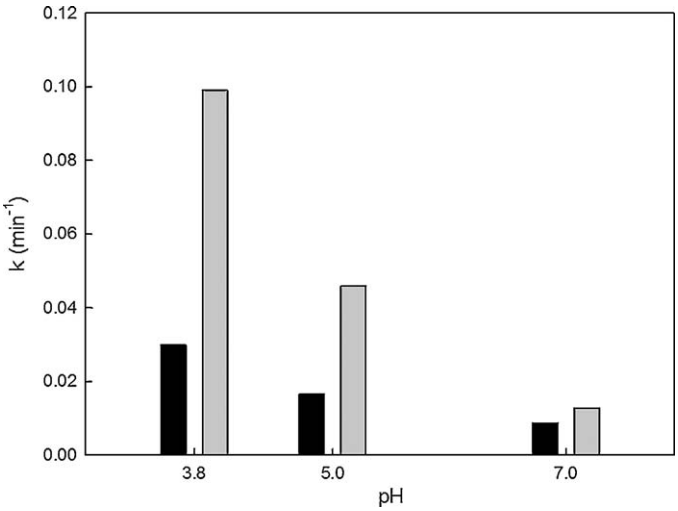

Fig. 1. Rate constant value dependence on pH and temperature for adsorption in 25 mM acetate–Tris–5 mM $CaCl_2$ buffer. Temperature: 6 °C (black bar) and 20 °C (gray bar).

ChTRP elution from CAB was carried out at 20 °C, after solid–liquid separation and exhaustive CAB washes with 25 mM acetate–Tris–5 mM $CaCl_2$ buffer until a negligible 280 nm absorbance measurement. For desorption, CAB were suspended in 25 mM acetate–Tris–$CaCl_2$ buffer and enzymatic activity and total protein were measured over time. The pH values assayed were: 3.8, 5.0 and 7.0 and $CaCl_2$ concentrations were: 100 mM, 300 mM, 500 mM and 1000 mM.

2.7. Polyacrylamide gel electrophoresis

SDS–polyacrylamide gel with 12% gel was performed using a BioRad Mini Protean II electrophoresis unit. 20 μL of the sample were boiled for 3–5 min. Gel was run at constant voltage (150 V). Then, gel was stained with Coomassie brilliant blue R-250 for 15 min and then destained in 40% methanol and 10% acetic acid.

3. Results and discussion

3.1. Commercial ChTRP adsorption

The experiments of batch kinetic adsorption of ChTRP onto alginate beads were carried out measuring the ChTRP activity in the supernatant. This allowed us to determine the kinetics of the process and the time required to reach the adsorption equilibrium. The experimental time course of residual ChTRP activity in the supernatant showed that the amount of ChTRP in the supernatant decreased with time. An exponential decay model is used to describe the adsorption kinetics, according to the following equation:

$$\ln(C - C_e) = -kt + i$$

where C_e is the activity of adsorbed ChTRP at equilibrium; C is the activity of adsorbed ChTRP in time; i is the integration constant; t is the time in minutes and k is the rate constant [15]. The values of the kinetic constant for the adsorption of commercial ChTRP at different pH and temperatures are shown in Fig. 1. The temperature has increased the value of the kinetic constant. The k was also dependent on pH of the medium. The lower the pH assayed the higher rate constant and the amount of ChTRP adsorbed onto the CAB. The best results were obtained at the lowest ionic strength (data not shown), maybe due to a coulombic interaction between the matrix and the enzyme. ChTRP concentration decreased sharply and adsorption was almost complete within 100 min.

The extent of the ChTRP (0.05%, w/w) adsorbed onto the CAB was highly dependent on the dry mass of CAB added to the system, as shown in Fig. 2. The ChTRP activity remaining in the supernatant