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Gamma trypsin: Purification and physicochemical characterization of a novel bovine trypsin isoform

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ABSTRACT

A novel bovine trypsin isoform was purified from commercial sample by ion exchange chromatography by Sephadex SP C50®. New isoform contains in addition of loss of N-terminus hexapeptide (as found in parent molecule β-trypsin) an intra-chain split between Lys-155 and Ser-156. The novel enzyme denominate γ-trypsin showed similar properties with α-trypsin isoform in polypeptide number chain (two chain), molecular masses (23,312 Da), secondary structure, hydrodynamic radius and others. In spite of enzymatic and structural similarities of both isoforms, γ-trypsin preferably has a lower rate formation from β-trypsin, a lower surface charge, but the γ-trypsin has a higher thermal stability than α-trypsin. Due to obtaining facility of purification of bovine trypsin isoforms from commercial font, and properties described above, this enzyme becomes an interesting alternative for the food industry, detergent and biocatalysis research.

1. Introduction

Proteases have been widely used for basic research and industrial applications [1] and about 50% of the total sale of enzymes in industries consist of proteases. Moreover, bovine viscera are a common by-product of the slaughterhouse and a possible enzyme source for biotechnological application. Trypsin plays an important role in the digestive process not only as proteolytic enzymes, but also because trypsin molecules activate many other digestive enzymes that are secreted as zymogens into the digestive intestinal tract. Trypsin (EC 3.4.21.4) belongs to the group of serine proteases; it specifically hydrolyses peptide bonds at the carboxyl side of lysine and arginine residues. Bovine trypsin is a proteolytic enzyme with a serine residue in the active site and an average molecular mass from 20,000 to 24,000 Da. Several closely related enzymes have also been identified, which are currently termed trypsin-like enzymes [2]. Trypsin and trypsin-like enzymes have been isolated from a several species as mammalian species such as humans and others animals [3–10] and in several species more than one isoforms of trypsin have been described. In humans as well as in the rat and the mouse, three isoforms have been isolated [11–13], in bovine species up to now three isoforms were described [14,15]. Commercial preparations of trypsin are a isoforms mixture and it were found to contain appreciable quantities of active form of trypsin as β-, α- and ψ-trypsin and others until not studied. In the present work, our objectives were to purify, to identify and to characterize the biochemical and physicochemical properties of novel trypsin isoform in order to infer about the profiling of biological conversion/degradation and to obtain useful information for application in industrial processes of γ-trypsin.

2. Materials and methods

2.1. Materials

Commercial bovine trypsin (EC 3.4.21.4) type I (Lot 8003), Tris-(hydroxymethyl) aminomethane, benzamidine-hydrochloride, α-cyano-4-hydroxycinnamic acid, N_α-benzoyl-DL-arginine-*p*-nitroanilide (BAPNA), N_α-benzoyl-L-arginine ethyl ester

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hydrochloride (BAEE), MES potassium salt were purchased from Sigma (St. Louis, MO, USA). SP-SEPHADEX-C50 matrix cation exchanger was purchased from Pharmacia Fine Chemicals INC (Uppsala, Sweden). Calcium chloride, sodium chloride, acetic acid, hydrochloride acid, dimethyl sulfoxide (DMSO), sodium phosphate monobasic buffer, glycine and sodium citrate were purchased from Synth (Diadema, SP. Brazil). Type I water was purified with a Barnstead nanopure Diamond™ Ultrapure water system (Barnstead International, Dubuque, USA).

2.2. Protein purification, determination of polypeptide number chains, molecular mass of trypsin isoforms and purity of γ-trypsin

2.2.1. Protein purification

Purification of trypsin isoforms was conducted according to [14] with minor modifications in the chromatographic parameters such as: mobile phase composition that was 100 mM Tris–HCl, pH 7.1 at 4 °C, 20 mM $CaCl_2$ and sample volume collected of 5 mL. The eluate was monitored by UV measurements at 280 nm and protein content was measured using molar coefficient extinction of 40,000 M^{-1} cm^{-1}. The tubes constituting each chromatographic peak of same trypsin isoforms were pooled and dialyzed against 1 mM HCl at 4 °C by a dilution factor of 10^6 times sample volume for 24 h and then lyophilized, aliquoted, and stored at 4 °C.

2.2.2. Determination of polypeptide number chains by SDS-PAGE, molecular mass of trypsin isoforms and purity of γ-trypsin by mass spectrometry

The polypeptide number chain from chromatographic fractions was determined by SDS-PAGE at 15% in reducing conditions according to [16], and stained by silver method [17]. Chromatographic peaks molecular masses and γ-trypsin purity were determined using mass spectrometry (MS) [18,19]. For MS experiments chromatographic fractions were dissolved in a saturated α-cyano-4-hydroxycinnamic acid matrix solution (1:3, v:v) spotted on to a MALDI target plate and dried at room temperature for 15 min. The average molecular mass was obtained in an AUTOFLEX III MALDI-Lift Tof-Tof (Bruker™) in linear positive mode, with external calibration (from 20 to 70 kDa) using the Protein Calibration Standard II (Bruker™). The Flex Analysis Software (Bruker Daltonics) was used to interpret mass spectra.

2.2.3. Edman degradation following of dansilation

In order to find intra-chain split in novel trypsin isoform were performed Edman degradation according to [20] followed by dansilation.

2.3. Assay of trypsin activity

The enzymatic activity, amidasic and esterasic were assayed according to [21], using the chromogenic synthetic substrate N_α-benzoyl-DL-arginine-*p*-nitroanilide hydrochloride (BAPNA) and N_α-benzoyl-L-arginine-ethyl-ester hydrochloride (BAEE). Chromogenic product absorption is read at 410 nm for BAPNA and 253 nm for BAEE. For each assay 4.5 μL of protein solution at 8.6×10^{-3} mM (stock solution at pH 3.0) was mixed with 12.5 μL of buffer and incubated at 310.15 K during 10 min and then was added 1 μL of substrate solution (BAPNA or BAEE) at 90 mM (solubilized in DMSO); thus, final protein concentration was set to 2.15×10^{-3} mM. After 15 min for amidasic activity and 35 min for esterasic activity, 4.5 μL of aqueous solution of acetic acid 60% (v/v) was added and then the absorbance was measured in spectrophotometer NanoDrop® 2000 Thermo. The specific activity was calculated using the following equation [22]:

$$\text{Specific activity} \quad (\text{U/mg}) = \left(\frac{\left((A \times \varepsilon \times 10^6 / T) \right)}{(x)} \right) \tag{1}$$

where A is the absorbance of chromogenic products formed, ε is the molar extinction coefficient 8800 M^{-1} cm^{-1} for *p*-nitroaniline and 1150 M^{-1} cm^{-1} for N_α-benzoil-L-arginine; and T (minute) is the time between substrate addiction and stopped reaction, and x is the amount protein at mg. One unit of activity (U) was defined as formation of 1 μmol of product per min. All measurements of activity were performing using 9×10^{-4} mg of protein.

2.3.1. pH activity profiles

For determination of γ- and α-trypsin pH activity profile, the amidasic and esterasic activities were tested over a broad range of pH from 2.5 to 9.0, using the different system buffers such as: 50.0 mM of Tris–HCl for pHs from 7.0 to 9.0; 50.0 mM of MES for pH 6.5; 50.0 mM of sodium citrate for pHs from 3.5 to 6.0 and 50.0 mM of glycine for pHs from 2.5 to 3.0. All buffer systems contained 20.0 mM $CaCl_2$. Enzyme activity measurements were performed according to item 2.3 and the data were expressed as the mean ± S.D. (n = 3).

2.3.2. Effect of $CaCl_2$ on enzymatic activity

The calcium concentrations effected over amidasic and esterasic activities of γ- and α-trypsin were tested adding: 0, 0.2, 0.4, 2, 20, 50, 100, 200 and 300 mM of $CaCl_2$ to protein solutions. All solutions contained 50.0 mM Tris–HCl buffer at pH 8.0. Enzyme activity measurements were performed according to item 2.3 and the data are expressed as the mean ± S.D. (n = 3).

2.3.3. Effect of NaCl on enzymatic activity

Sodium chloride effect concentration over amidasic activity of γ- and α-trypsin was tested using a solution of 100.0 mM Tris–HCl buffer at pH 8.0; with addition of 0, 0.2, 0.4, 2, 20, 50, 100, 200, 300, 400, 500 and 600 mM of NaCl into protein solutions. The pH 8 was maintained constant for all conditions tested. Enzyme activity measurements were performed according to item 2.3 and the data were expressed as the mean ± S.D. (n = 3).

2.3.4. Thermal stability

Thermal stability of γ- and α-trypsin [23] was determined at different temperatures, from 298.15 K to 348.15 K for two methods: First described here and second in circular dichroism (CD) 2.6. item. Thermal stability was performed measuring the decay of activity to reduce it to 50% using BAPNA substrate. The experiment was performed during 90 min; every 10 min was collected 4 μL of solution that was transferred to tubes containing 2.4 μL of an aqueous solution of acetic acid (60% v/v) and then absorbance at 410 nm was measured. Experimental conditions were: 42.2 μL of 50 mM of Tris–HCl buffer at pH 8.0; with 20.0 mM $CaCl_2$, 5 μL of protein at 0.02 mM and 2.8 μL of BAPNA substrate at 90 mM. The pH 8 was maintained constant for all conditions tested and for this purpose pH solutions were adjusted considering the dpK_a/dT of Tris–HCl (−0.028/K) for each temperature. The data were expressed as the mean ± S.D. (n = 3).

2.4. Hydrodynamic radius

Hydrodynamic radius was measured by Dynamic light scattering (DLS) at 830 nm according to [24] using a Protein Solution® Dyna Pro®. Measures were performed for γ- and α-trypsin at 0.04 mM in 30 mM sodium phosphate monobasic at pH 3.0 at