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Cloning and expression of ostrich trypsinogen: an avian trypsin with a highly sensitive autolysis site

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Abstract

One of ostrich (*Struthio camelus*) trypsinogen genes was cloned from pancreatic cDNA. Its amino acid sequence compared to known trypsin sequences from other species shows high identity and suggests that it is a member of the phylogenetically anionic trypsinogen I subfamily. After cytoplasmic over expression in *Escherichia coli* and renaturation, the activation properties of ostrich trypsinogen were studied and compared to those of human trypsinogen 1 (also called as human cationic trypsinogen). Ostrich trypsinogen undergoes bovine enterokinase activation and autoactivation much faster than human trypsinogen 1 and exhibits on a synthetic substrate a somewhat higher enzymatic activity than the latter one. The most interesting property of ostrich trypsin is its relatively fast autolysis that can be explained via a mechanism different from the common mechanism for rat and human 1 trypsins. The latter proteases have a site, Arg117–Val118, where the autolysis starts and then goes on in a zipper-like fashion. This is absent from ostrich trypsin. Instead it has a couple of cleavage sites within regions 67–98, including two unusual ones, Arg76–Glu77 and Arg83–Ser84. These appear to be hydrolysed fast in a non-consecutive manner. Such an autolysis mechanism could not be inhibited by a single-site mutation which in humans is proposed to lead to pancreatitis.

Keywords: Ostrich; Trypsinogen; Autolysis

1. Introduction

Kühne [1] described first, more than 130 years ago, that cattle pancreatic juice possessed the property to digest proteins. He proposed that this property was due to an 'enzyme' that he named 'trypsin'. It was also reported by Kühne [1] that the extract from freshly removed cattle pancreas exhibited only a low proteolytic activity, but it was increasing when the juice was allowed to stand. Upon longer standing, however, the proteolytic activity started to decrease. This was the first description of autoactivation of the inactive form and autolytic inactivation of the active form of trypsin, respectively [1]. Since then trypsinogen (the inactive, proenzyme form of trypsin) and trypsin have become prototypes of investigating many aspects of protease action, including the molecular mechanisms of proenzyme autoactivation and the autolysis of the active

Abbreviations: *N*-CBZ-GPR-pNA, *N*-benzyloxycarbonyl-Gly-Pro-Arg-*para*-nitroanilide; oligodT, oligonucleotide containing only thymine specific for the poly-adenine tail of the mRNA; LB, Lauria-Bertani broth; MUB, 4-methylumbelliferone; MUGB, 4-methylumbelliferyl-4-guanidinobenzoate; HuTg-1, human trypsinogen 1; HuTr-1, human trypsin 1; IPTG, isopropyl-thio-galactose; GdnHCl, guanidine-hydro-chloride; EDTA, ethylene diamine tetraacetic acid; PCR, polymerase chain reaction; STI-agarose, soybean trypsin inhibitor linked to agarose; Tris–HCl, α,α,α-Tris–(hydroxymethyl)-methylamine hydrochloride; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; ctg, chymotrypsinogen; sp, serin protease; tg, trypsinogen; tr, trypsin; $NH_4(HCO_3)$, ammonium–hydrogen–carbonate

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protease [2–5]. Much of the fundamental knowledge about the structural basis of substrate specific serine protease action has also been derived from studies on mutants and variants of trypsin provided by either protein engineering [6,7] or natural evolution. As to the latter alternative, recent examples are human pancreatic mesotrypsin that is highly resistant to canonical trypsin inhibitors [5,8], and crayfish trypsin that, on contrary, shows extremely high affinity towards inhibitors that does not inhibit mammalian trypsins [9]. Thus, it's still a rewarding strategy to explore structure–activity relationships in trypsins from evolutionary significant new species [10]. Ostrich belongs to one of these species representing the connecting taxon (Ratite) between birds and reptiles [11]. We thought to clone the cDNA of ostrich trypsinogen, determine its sequence and express the zymogen in *Escherichia coli* in order to compare its biochemical properties to those of some already well-characterized trypsins.

These studies of ostrich trypsinogen provided us with new insight into the mechanism of trypsin autolysis. Our results show that ostrich trypsin has a unique, multi-cleavage site autolysis region that assures fast autolysis of trypsin in both the intestine and pancreas of ostrich. Such an autolysis mechanism cannot simply be blocked by a single-site mutation of trypsin causing the accumulation of the protease in the pancreas.

2. Materials and methods

2.1. Cloning

Total RNA was isolated from ostrich pancreatic tissue (10 mg), stored in RNeasy solution (Qiagen) by using TRI Reagent (Sigma) according to the manufacturers instruction. cDNA was polymerized with oligodT from the isolated RNA using the Fermantas Revert Aid H Minus First Strand cDNA synthesis kit. The ostrich trypsinogen gene was amplified from the cDNA pool with odT and 5′ end [5′GTN CCN GGN GAY GCN GAY GAY GAY AAR ATH 3′] degenerated primer designed on the basis of the N-terminal amino acid sequence of ostrich trypsinogen [12]. The PCR product was ligated into a pKS+ (Stratagen) vector using TA cloning, and sequenced from both directions with T7 and T3 plasmid specific primers, by automated dideoxy sequencing (ABI Prism) using a Big Dye Terminator kit. After obtaining the DNA sequence the gene was amplified with specific primers. For the 5′ end, 5′C GAA GCT TTG CCC GGT GAT GCC GAT GAC GAC AAG ATC G3′ was used. This contains a *Hin*dIII restriction site downstream from the start codon that extends the known propeptide amino acid sequence [12] with an extra MSTQAL sextapeptide at the N-terminus. Because of the latter extension the expressed protein is although differing from the native pretrypsinogen in the signal peptide, should activate similarly to the native trypsinogen, and will produce a native trypsin. For the 3′end, 5′CGG GAG CTC ATC GAG GCA TCA GTA GGC 3′, including a TAG stop codon and an *Sac*I restriction site was applied. The PCR product was ligated into a pET17c cytoplasmic expression vector (Novagen), for use in *E. coli*, and sequenced.

2.2. Expression and refolding

Ostrich trypsinogen was expressed in BL21 DE3 pLysS (Stratagen) cells into the cytoplasm as an inclusion body. Cells were grown in 500 ml LB broth containing 100 μg/ml ampicillin (Sigma) at 37 °C in a shaking incubator to an absorbance of 0.8 at 600 nm and induced with IPTG (Sigma) (final concentration of 100 μg/ml) for 4 h. Cells were harvested by centrifugation at 14300×*g* for 30 min at 4 °C (Beckman J2-MC), redissolved in 50 ml buffer [50 mM Tris–HCl (Sigma) and 20 mM EDTA (Sigma), pH 8.0], frozen at −20 °C and lysed by thawing. The inclusion bodies were isolated from the cell lysate after adding DNAse (Sigma) (5 μg/ml final concentration), centrifuged (20000×*g*, Beckman J2-MC) and washed twice with the abovementioned buffer. Fifty milligrams of the isolated inclusion body was dissolved in 5 ml 6 M GdnHCl containing 0.1 M Tris–HCl, 2 mM EDTA and 48 mM DTT, pH 8.5, flushed with nitrogen. The dissolved inclusion bodies were incubated at 37 °C with shaking for 30 min. The solution was added to a 45 ml buffer [6 M GdnHCl, 1 mM cystine, 1 mM cysteine in 0.1 M Tris–HCl and 2 mM EDTA, pH 8.5] at 4 °C. The whole refolding mixture (50 ml) was placed in a dialysis tube, and 6 M GdnHCl was dialysed out against 250 ml [1 mM cystine, 1 mM cysteine, 0.1 M Tris–HCl and 2 mM EDTA, pH 8.5] buffer so the final concentration of GdnHCl became 1 M. Thereafter the sample was dialysed into 2.5 mM HCl and stored at −20 °C. HuTg-1 was expressed and refolded as described previously [3].

2.3. Purification

Ecotin column chromatography was employed for zymogen purification [13]. Purified zymogen, activated by trypsin-free bovine enterokinase (Sigma) (purified on an ecotin column) with a final concentration of 0.0125 mg/ml for 60 min at 37 °C in buffer [50 mM Tris–HCl, 10 mM $CaCl_2$ and 0.5 M NaCl, pH 8] was loaded onto a 1 ml STI-agarose column (Sigma). The column was washed with distilled water and eluted with 20 mM HCl. The purities of ostrich trypsinogen and trypsin are illustrated by gel electrophoresis in the presence and absence of a reducing agent (Fig. 1). The protein concentration was determined by measuring the absorbance at 280 nm using theoretical molar extinction coefficients for HuTg-1 and ostrich trypsinogen (ε=36160 and ε=38840, respectively).

2.4. Kinetic measurements

The concentration of the purified zymogens after activation with enterokinase (HuTr-1 and ostrich trypsin)