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Comparison of natural and recombinant clitocypins, the fungal cysteine protease inhibitors

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Abstract

A member of the cysteine protease inhibitor clitocypin gene family from basidiomycete *Clitocybe nebularis* was expressed in *Escherichia coli*. Following careful optimization of the expression procedure the active inhibitor was purified from inclusion bodies and its properties examined and compared to those of the natural clitocypin. The CD spectrum of recombinant clitocypin was similar to that of natural clitocypin, indicating that protein was properly refolded during purification. In spite of some differences in primary structure, structural, functional and immunological equivalence was established. Kinetic analyses of the natural and recombinant clitocypins were performed. Both clitocypins inhibited a range of cysteine proteases to a similar extent, and demonstrated an unusually broad inhibitory spectrum, including distantly related proteases, such as papain and legumain, belonging to different protease families. The homogenous, biologically active recombinant clitocypin is obtained at levels adequate for further structure-function studies.

Keywords: *Clitocybe nebularis*; Mushroom; Basidiomycete; Clitocypin; Cysteine protease inhibitor; Heterologous expression; Cysteine protease; Cathepsins; Legumain; *Escherichia coli*

Peptidases and their inhibitors are present in all life forms under normal physiological and pathological conditions. Since peptidases are potentially very damaging, the proper control of their activity by specific inhibitors is very important. In higher organisms deregulated proteolytic activity may well contribute to a number of disease conditions [1], and in bacteria [2] and fungi [3] proteases are actively involved in pathogenesis and virulence. Not surprisingly, the growing interest in naturally occurring peptidase inhibitors is driven by their many possible applications in medicine, agriculture and biotechnology.

Numerous individual peptidase inhibitors and inhibitor families have been reported from practically all eukaryotic organisms except fungi, where their number is still limited [4]. Higher fungi or basidiomycetes, commonly called mushrooms, have been largely overlooked in the search for peptidase inhibitors, except for the detection of inhibitors of serine and metallo-peptidases in basidiocarp extracts [5,6]. Only a few peptidase inhibitors have been adequately purified and described from mushrooms. These include low molecular mass inhibitors of endogenous serine proteinase A from *Pleurotus ostreatus* [7], trypsin/chymotrypsin inhibitor from *Lentinus edodes* [8] and a specific cysteine protease inhibitor from *Clitocybe nebularis* [9]. The latter has been given the name clitocypin (*C. nebularis* cysteine protease inhibitor). It is unusually stable to heat and denaturing agents, with completely reversible unfolding [10], and resists proteolytic degradation. Clitocypin was the first inhibitor of cysteine proteases to be characterized from fungi and displayed no significant similarity at the level of its 152 amino acid sequence to any known peptidase inhibitor or other proteins in the databases, including genomic [11]. It has been assigned as the sole member of the I48 inhibitor family in the MEROPS inhibitor classification [4].

Originally we observed that protein and cDNA derived sequences differed in seven amino acid residues [9]. Subsequent characterization of several cDNA and genomic

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clones encoding the clitocypin gene led to the identification of multiple nucleotide and predicted peptide sequences. They are accounted for by heterogeneity in the clitocypin gene [11].

This microheterogeneity of the inhibitor from the natural source led to the need for a reproducibly uniform source for the active inhibitor. In this paper, we describe the cloning and high yield expression of recombinant clitocypin in *Escherichia coli*. We focus on a more detailed comparison of the properties of the recombinant (rClt) and natural (nClt) clitocypins and extend the number of cysteine proteases for which we have determined binding specificity.

Materials and methods

Enzymes. Human cathepsin H (EC 3.4.22.16) and cathepsin L (EC 3.4.22.15) were purified as described [12]. 2× crystallized papain (EC 3.4.22.2) was further purified by affinity chromatography [13]. Recombinant human cathepsin K [14] and caspases 3, 6 and 7 [15] all expressed in *E. coli* and legumain isolated from pig kidney cortex [16] were provided by prof. Boris Turk (our laboratory).

Isolation of natural clitocypin. Basidiocarps or fruiting bodies of *C. nebularis* (also classified as *Lepista nebularis*) were harvested in October 2001 on the same location as previously reported [9] and frozen at –20 °C. The inhibitor was purified as described [10] with hydrophobic and ion exchange chromatography as the main steps. The purity of the natural clitocypin was analyzed by SDS–PAGE[1] under denaturing conditions.

Construction of expression plasmids. For expression of recombinant clitocypin the previously reported cDNA clone, Kras-cDNA, [11] (GenBank Accession No. DQ150588) was used. This cDNA clone was used as template for PCR amplification with Pfu DNA Polymerase (Promega) and primers which introduced *Nde*I and *Bam*HI restriction sites to the 5′ and 3′ ends of the insert, respectively. The resulting product was cloned into pGEM-T Easy vector (Promega) and re-sequenced to ensure lack of mutations. After *Nde*I/*Bam*HI (New England Biolabs) digestion of both insert and vectors the insert was subcloned into pET3a and pET11a vectors (Novagen) to generate recombinant proteins without tags. Both expression vectors were transformed into BL21(DE3) Star (Invitrogen), and BL21(DE3) pT-Trx [17] strains of *E. coli* and the expression vector pET3a-rClt with insert was transformed also into the *E. coli* strain BL21(DE3) pLysS (Novagen).

Expression and purification of recombinant clitocypin in E. coli. BL21(DE3) Star strain of *E. coli* transformed with expression vectors pET3a-rClt and pET11a-rClt was grown in Luria-Bertani (LB) medium supplemented with 100 μg/ml ampicillin at 37 °C, and transformed *E. coli* strains BL21(DE3) pT-Trx and BL21(DE3) pLysS were grown in LB medium supplemented with 100 μg/ml ampicillin and 34 μg/ml chloramphenicol at 37 °C. When optical density at 600 nm reached 0.4–0.5, the inducer isopropyl-β-D-thiogalactopyranoside was added to a final concentration of 0.4 mM for strains transformed with pET3a construct and 1 mM for strains transformed with pET11a construct. Cells were grown for an additional 8 h. For hourly monitoring of protein expression a sample was collected, cells harvested by centrifugation for 10 min at 6000*g*, resuspended in buffer A (50 mM Tris–HCl, pH 8, 0.1% Triton-X-100, 2 mM EDTA) and sonicated at 4 °C. Samples were then examined by polyacrylamide gel electrophoresis in the presence of SDS.

For expression of recombinant clitocypin the pET3a construct transformed into BL21(DE3) pT-Trx strain of *E. coli* was used. The cells were grown in LB medium supplemented with 100 μg/ml ampicillin and 34 μg/ml chloramphenicol at 37 °C until optical density at 600 nm reached 0.4–0.5, when isopropyl-β-D-thiogalactopyranoside was added to a final concentration of 0.4 mM and cells were grown for an additional 4 h. Cells were then harvested by centrifugation, resuspended in buffer A, frozen and thawed once, then sonicated at 4 °C. The insoluble fraction was separated by centrifugation (10,000*g*, 15 min) redissolved in the same buffer containing 8 M urea and solubilized by stirring for 10 h at 4 °C. The remaining insoluble material was removed by centrifugation and the supernatant concentrated on a UM-3 membrane (Amicon). A Sepharose S200 column (4 × 110 cm), equilibrated with 0.1 M Na-acetate buffer, pH 5.5 containing 0.3 M NaCl, was used for gel filtration. Homogenous inhibitory fractions were pooled and concentrated to ≈1 mg/ml.

Production of polyclonal antibodies. Purified recombinant clitocypin was used for the production of polyclonal antibodies in rabbit (White New Zealand). 600 μg of the immunogen in Freund's complete adjuvant was injected, following the standard protocol and schedule.

Protein concentration estimation. Protein concentration in solution was determined by absorbance at 280 nm, using a Perkin–Elmer UV–Vis spectrophotometer λ18. The protein concentration of clitocypin was determined using molar absorbance coefficient of 22.900 $M^{-1} mol^{-1}$, calculated from the amino acid sequence (Q9P4A2) [9].

IEF and SDS–PAGE analysis. Isoelectric focusing and SDS–PAGE were performed using the Fast System (Pharmacia-LKB), according to the manufacturer's instructions. Precast pH 3–9 gradient gels were calibrated with marker proteins with pI values ranging from 3.5 to 9.3 (Amersham Pharmacia Biotechnology). For SDS–PAGE precast 8–25% gradient gels were used together with molecular weight markers. Alternatively, proteins were separated in 12% (w/v) polyacrylamide gels according to Laemmli [18], using a mini-Protean II apparatus (Bio-Rad). Unless otherwise indicated samples were denatured by boiling for 10 min in equal volumes of buffer containing 5% SDS without reducing agent. Proteins were visualized by Coomassie Brilliant Blue R-250 staining.

[1] *Abbreviations used:* AFC, 7-amino-4-trifluoromethylcoumarin; HPLC, high pressure liquid chromatography; AMC, 7-amino-4-methylcoumarin; PAGE, polyacrylamide gel electrophoresis; SDS, sodiumdodecylsulphate; Z, benzyloxycarbonyl.