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Bacterial expression of human cysteine proteinase inhibitor stefin A

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Stefin A, a cysteine proteinase inhibitor of the cystatin superfamily, has been found to be most abundant in epidermal cells. In order to determine its cellular function, we have expressed human stefin A in *Escherichia coli* using plasmid expression vectors under the control of bacteriophage T7 RNA polymerase. The heat-stable, antibody-positive bacterial product was isolated using a papain-Sepharose affinity column and was shown to inhibit two cysteine proteinases, papain and human cathepsin B. Recombinant stefin A may have commercial and therapeutic potential in situations requiring inhibition of cysteine proteinase activities, and in cosmetics, as an ingredient in skin creams.

Gene expression system; DNA, recombinant; Stefin A; Cystatin A

1. INTRODUCTION

Proteinases are implicated in a variety of cellular functions, and their occurrence emphasizes the importance of proteolysis in the control of cellular processes [1]. We have been interested in cysteine proteinases such as cathepsin B because of their potential role in diseases including tumor metastasis [2]. For the four groups of proteinases, the serine, cysteine, aspartic and metallo-types, many proteinase inhibitors are known [3]. The protein proteinase inhibitors for cysteine proteinases are members of the cystatin superfamily. With a wide phylogenetic distribution, the members are divided into three families: stefins, cystatins and kininogens; at least one human hereditary disease is caused by a cystatin mutation [4]. The stefins are low-molecular-weight, carbohydrate-free, thermostable protein molecules. Human and rat stefins have been characterized, with stefin B demonstrating a general tissue distribution and stefin A found predominantly in epidermal keratinocytes and polymorphonuclear leucocytes [5,6]. Our study on keratinocyte gene expression led us to human stefin A (also known as cystatin A) complementary DNA clones [7]. Although a mouse stefin A cDNA clone has been independently reported [8], to our knowledge, ours are the only human clones. Since it took 80 l of fresh human blood for the first characterization of stefin A [9], we decided to make the recombinant protein for functional studies.

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2. MATERIALS AND METHODS

2.1. *Construction of expression vectors*

Previously, we used the pET series of expression vectors for human cathepsin B [10], and we did the same for stefin A. The *Eco*RI-*Bam*HI 1.5 kb fragment from stefin A cDNA clone 242 [7], first subcloned in pUC9, was isolated and cut with restriction enzymes *Nla*IV and *Sau*3AI. This generated a 384 bp fragment containing the complete stefin A coding region. After Klenow fill-in and *Bam*HI linker addition, the insert was cloned into pBR322 and transferred to pET3a, pET3b and pET3c expression vectors (in *E. coli* host HMS174). Orientation of cDNA insertion was determined based on *Hinc*II restriction pattern. Six clones, designated pMC31 through pMC36, were selected to correspond to stefin A cDNA insert in the correct and opposite direction for the three vectors (see fig.1). These were used to transform the expression host BL21(DE3)pLysS and tested for stefin A production. Bacteria were grown in nutrient broth containing ampicillin and chloramphenicol; fusion protein production was induced by 1 mM IPTG (isopropyl-β-D-thiogalactoside), and cells were pelleted and frozen at −70°C.

2.2. *Isolation of recombinant protein*

For affinity chromatography, the bacterial lysate (pMC33) was sonicated in buffer (10 mM Tris-HCl, pH 7.6) containing 2 mM EDTA, the debris was removed by centrifugation, and the sample heat-treated (100°C for 15 min). After centrifugation, the heat-soluble fraction was loaded onto a papain-Sepharose column (from Pierce, pretreated with buffer containing 0.5 M NaCl) [11]. After flow-through of unbound material, the column was washed with buffer containing 3 M KCl, and bound material was eluted with 50 mM Na_3PO_4, pH 11.5, containing 0.5 M NaCl. The collected fraction, neutralized with 1 M HCl, was found to be stefin A fusion protein by gel analysis. Protein quantitation was done by the BCA (bicinchoninic acid) method (from Pierce).

2.3. *Assays of proteinase inhibitors*

The peptide Z-Arg-Arg-AFC (7-amino-4-trifluoromethylcoumarin, from Enzyme Systems Products) was used as a substrate for cathepsin B [12]. Dissolved in dimethyl formamide at 10 mg/ml, it was diluted to 25 μM in buffer (0.2 M sodium phosphate, pH 6) con-

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taining 5 mM dithiothreitol and 1 mM EDTA. For a simple assay in microtiter plates, bacterial lysates (typically 30 μl) or column eluates were pretreated with human liver cathepsin B (from Calbiochem, diluted to 10 μg/ml, typically 5 μl) for 10 min at room temperature, and then 50 μl of substrate was added. After incubation for 15 min at 37°C, the plate was viewed under a UV transilluminator (Fotodyne). The peptide AFC has blue fluorescence, whereas the free AFC has green fluorescence.

For inhibition constants, the activities of papain (Sigma; repurified on Sephadex G-50) and cathepsin B (Calbiochem) were determined in the presence of appropriately diluted inhibitor and 0.1 M acetate buffer, pH 5.5, or citrate phosphate buffer, pH 6.2, respectively, following a 10 min activation at 37°C with 5 mM dithiothreitol and 2 mM EDTA. The assays were terminated by the addition of 100 mM sodium monochloroacetate, containing 30 mM sodium acetate and 70 mM acetic acid [13].

2.4. *Western blot analysis*

Protein was transferred from gel to filter (0.2 μm nitrocellulose from Sartorius) using Western blot technique [10]. After shaking for 1 h at room temperature with TTBS (Tris-buffered saline, 20 mM Tris-HCl, pH 7.5, 0.5 M NaCl containing 0.05% Tween-20), the filter was treated with 1:2000 dilution of rabbit antiserum to rat low-molecular-weight cysteine proteinase inhibitor [14]. The antiserum was precleared with an unrelated bacterial lysate before use (using pMC4) [10]. After overnight incubation, the filter was washed in TTBS and treated for 1 h with a secondary antibody (1:2000 dilution of affinity purified goat anti-rabbit antibody conjugated to horseradish peroxidase, from Southern Biotechnology). After washings in TTBS and TBS, the filter was developed with the chromogenic substrate Indophane Red (from Vio-medics).

3. RESULTS

The coding segment of stefin A from cDNA clone 242 [7] was ligated into the expression vector, resulting in the plasmid designated as pMC33 (fig.1). A control plasmid, with the stefin DNA segment cloned in the opposite orientation, was named pMC34. In the appropriate bacterial host, recombinant stefin protein was detected. Since it is a fusion protein containing T7 ϕ10 protein amino-terminal sequence (11 amino acid residues), *Bam*HI linker sequence (3 residues), 5′ sequence of the cDNA (12 residues), and the stefin A coding region (98 residues), its apparent molecular mass was 16 kDa by SDS-polyacrylamide gel analysis (fig.2A). The calculated value was 14313 Da; the larger apparent molecular weight may result from a change in mobility due to protein configuration change). The protein was inducible by IPTG, but there was a basal

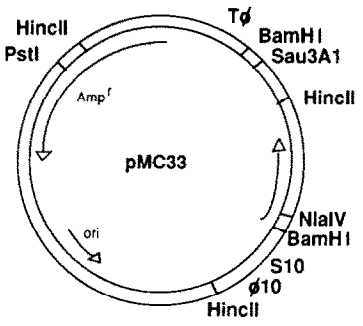

Fig.1. Human stefin A cDNA plasmid pMC33.

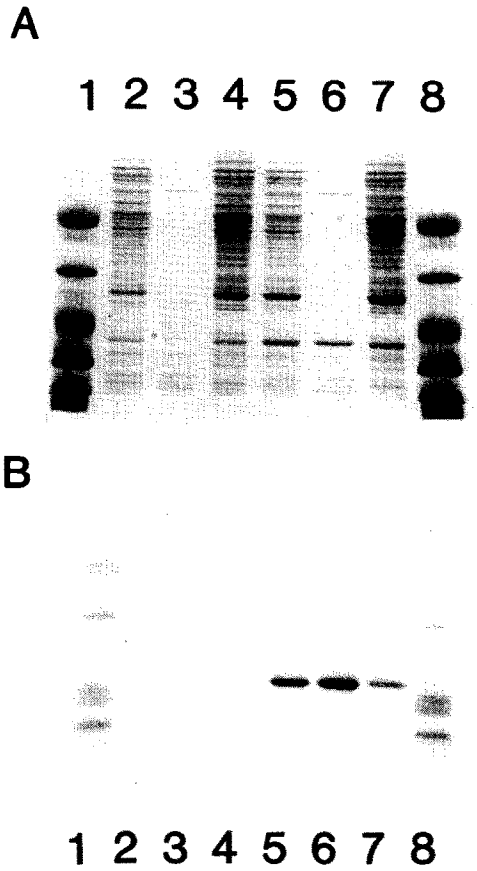

Fig.2. SDS-polyacrylamide gel electrophoresis and Western blot of recombinant stefin A. Lanes: 1 and 8, molecular mass standards (prestained standards from Bethesda Research Laboratories, the six bands are 43, 29, 18, 14, 6 and 3 kDa in size); 2–4, from plasmid pMC34; 5–7, from pMC33. The three lanes denote total bacterial lysate, heat soluble and insoluble fractions. For 12.5% minigel, 10 μg protein was loaded for lanes 2 and 5. (A) Coomassie blue-stained gel. (B) Antibody-stained nitrocellulose blot.

level of expression. (Data not shown. This aspect differs from our experience with cathepsin B, in which we found no expression without induction.) The protein was heat-stable and was found in the soluble fraction of bacterial lysate after heat treatment (fig.2A), an observation similar to recombinant oryzacystatin, a cysteine proteinase inhibitor from rice [15]. Recombinant human stefin A was characterized by Western blotting using a rabbit antiserum to rat low molecular weight cysteine proteinase inhibitor and was found to be immunoreactive (fig.2B). (This antiserum was positive by slot blotting for both purified human stefins A and B. Data not shown.)

Recombinant human stefin A was assayed for its action on cysteine proteinases. It was found to bind to a papain-Sepharose column, therefore we used this as a single-step procedure for inhibitor isolation. For a sample run on a 2 ml column: a starting material containing 29 mg of bacterial protein yielded a 0.26 mg recombinant protein eluate. (This may be a low

estimate due to the saturation of column capacity.) Both bacterial lysate (from pMC33) and eluted stefin A were found to inhibit human cathepsin B activity, using Z-Arg-Arg-AFC as substrate for proteolysis, whereas bacterial lysate from control samples (such as pMC34) were ineffective (data not shown). Furthermore, inhibition constants of the recombinant stefin A were in the nanomolar range for papain and cathepsin B (using Z-Phe-Arg-AMC, 7-amino-4-methylcoumarin, and Z-Arg-Arg-AMC as substrate, respectively, table 1), similar to those of purified stefin A [16]. Thus, recombinant human stefin A fusion protein was functionally active.

4. DISCUSSION

Previous attempts to express stefins in bacteria involved synthetic genes (by chemical synthesis based on known amino acid sequences). Recombinant human stefin A [17,17a], human stefin B [18,18a] and rat stefin A [19] have all been reported. In contrast, our result is the first that was derived from human stefin A cDNA. One would expect that expression from synthetic genes should give a larger yield because of the optimization of codon usage for bacteria. However, our yield was much higher than that of the human stefin A synthetic gene (100 μg from 6 g bacteria), and, in the latter, there was no additional synthesis after induction [17]. This difference may be due to the choice of expression vectors.

Recombinant stefin A may be used to investigate its cellular and physiological functions. For example, a chicken cystatin has been shown to stimulate mouse fibroblast growth [20]. Recombinant stefin A may have potential therapeutic and commercial values, as in the skin disease psoriasis, in which a defective stefin A has been detected [21], and in cases such as carcinogenesis and metastasis [2,22]. In this regard, we have recently isolated normal stefin B and defective stefin A from human sarcomas [16].

High concentrations of stefin A selectively localized in epithelial cells and polymorphonuclear leucocytes suggest a defensive role for this inhibitor. Anti-viral and anti-bacterial capabilities of cystatins have been described [23,24]. There is an abundance of stefin A mRNA in chemically induced mouse skin papillomas but a decrease in mRNA with progression to carcinomas [8]. We have seen an increase in stefin A mRNA expression in UV light-irradiated human keratinocytes [7]. Thus, it may be therapeutically beneficial to include stefin A as a component of skin creams in cosmetics (for use by individuals with prolonged exposure to the sun). Natural proteins, such as collagen, have been used as ingredients in cosmetics [25].

Table 1

Inhibition constants ($K_{i_{app}}$) for the interaction of recombinant human stefin A with papain and human liver cathepsin B

Enzyme	$K_{i_{app}}$ (nM)	
	A	B
Papain	0.20	0.23
Cathepsin B	13.3	10.3

The inhibition constants were calculated from the Henderson plot of V_0/V_i against $[I]/1 - V_i/V_0$, where V_0 = activity in the absence of the inhibitor, V_i = same but in the presence of the inhibitor at several dilutions, [I] = the concentration of the inhibitor. The slope of the line through the points = K_i; column A in the table. Alternatively, the inhibition constants were calculated according to the procedure of Nicklin and Barrett [13]; column B in the table

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