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Properties and nature of a cysteine proteinase inhibitor located in keratohyalin granules of rat epidermis

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The p*I* 4.7, 14.5 kDa hematoxylin-stainable protein (HSP) from rat epidermis inhibited the activities of the cysteine proteinases papain, ficin, cathepsins B, H and L with similar inhibitory characteristics as recombinant cystatin-α. Proteinases of other classes were not inhibited. The inhibitory activity of HSP was heat stable in the wide pH range of 3.0–10.0. Polyclonal antibodies against HSP cross-reacted with cystatin-α and the molecular mass of HSP was similar to that of cystatin-α, though its isoelectric point was different. The in vivo location of both HSP and cystatin-α is on keratohyalin granules in epidermis as detected by indirect immunofluorescence technique using individual antibodies. Therefore it is highly probable that HSP is a cystatin-α derivative or a very similar proteinase inhibitor belonging to a family of cystatins.

Cysteine proteinase inhibitor; Keratohyalin granule; Hematoxylin stainable protein; Cystatin-α

1. INTRODUCTION

Cysteine proteinase inhibitors have been isolated from various mammalian tissues and are divided into 3 groups of families [1]. Some cysteine proteinase inhibitors were isolated from human and rat skins, and characterized [2,3]. One of them, cystatin-α, which belongs to family 1, has a low molecular weight and has been found in the skin in high levels, especially in the epidermal cells [4]. However, its in vivo location has not been clarified yet. Recently, we have isolated an acidic, hematoxylin-stainable protein component (HSP) of keratohyalin granules (KHG) [5,6]. As shown in Table I, the amino acid composition of HSP was found to be similar to that of cysteine proteinase inhibitors isolated by Takeda et al., named TPI(1) [7] and Takio et al., named rat epidermal TPI [8]. This finding suggested that HSP could be a member of the cystatin superfamily and may inhibit cysteine proteinases.

In this work, the inhibitory properties of HSP were investigated and found to be similar to those of cystatin-α. Further similarities of HSP to cystatin-α were found in molecular mass, immunological reaction and tissue distribution.

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Abbreviations: HSP, hematoxylin stainable protein; KHG, keratohyalin granules; PoAb, polyclonal antibody; HE stain, hematoxylin-eosin stain; K_i, inhibition constant

2. MATERIALS AND METHODS

2.1. *Materials*

Papain, ficin, chymotrypsin, thermolysin, casein and *p*-nitroanilide substrates were purchased from Sigma (USA). Trypsin was obtained from Difco (USA). Rat cathepsins B, H and L were purified as described [9–11]. Aminomethylcoumarin substrates were purchased from Peptide Institute (Japan). Superose 12 and Mono-Q columns and ampholines were ordered from Pharmacia-LKB (Sweden). Lowicryl K4M embedded kit was obtained from TAAB Laboratories (UK). Recombinant cystatin-α and polyclonal antibodies against it were kindly provided by Dr T. Ohshita (Division of Enzyme

Table I

Comparison of amino acid composition

	HSP [6]	TPI(1) [7]	Rat epidermal TPI [8]
Half-Cys	0	0	–
Asx	12.0	12.4	11.3
Thr	7.3	8.7	6.4
Ser	3.6	5.3	3.9
Glx	14.3	15.1	13.8
Pro	3.3	6.9	4.1
Gly	10.0	9.6	9.4
Ala	4.5	3.9	5.0
Val	8.0	9.7	8.7
Met	2.6	2.7	2.7
Ile	2.2	2.7	2.9
Leu	8.7	9.3	8.8
Tyr	2.9	2.3	2.9
Phe	4.0	4.1	4.0
His	1.2	1.5	1.3
Lys	13.7	13.7	11.6
Arg	3.2	3.0	3.3
Total	101.5	110.9	103

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Table II

Purification of the HSP from newborn rat epidermis

Step	Total protein (mg)	Activity[a]		Yield (%)
		(U)	(U/mg)	
Tris-HCl epidermal extract	740	155.4	0.21	100
Isoelectric focusing	125	46.3	0.37	29.8
Superose 12	47.6	39.0	0.82	25.1
Mono-Q	23.1	23.6	1.02	15.2

[a] The inhibition was expressed as mg of papain inhibited by 1 mg of inhibitor (U/mg)

Chemistry, Institute for Enzyme Research, The University of Tokushima) [12].

2.2. *Purification of HSP and production of polyclonal antibodies*

Rat HSP was purified to homogeneity by 2-dimensional electrophoresis as described previously [6]. Three-day-old rat (Sprague-Dawley strain) epidermal extract in 50 mM Tris-HCl (pH 7.4) was subjected to preparative isoelectric focusing, Superose 12 gel filtration and Mono-Q anion-exchange column chromatography. PoAbs against thus purified HSP were produced in rabbits.

2.3. *Assay of inhibitory activity*

The inhibitory activities of HSP and the recombinant cystatin-α [8,12] on papain, ficin [7], pepsin [13] and thermolysin [14] were assayed using casein or authentic *p*-nitroanilide substrates; the absor-

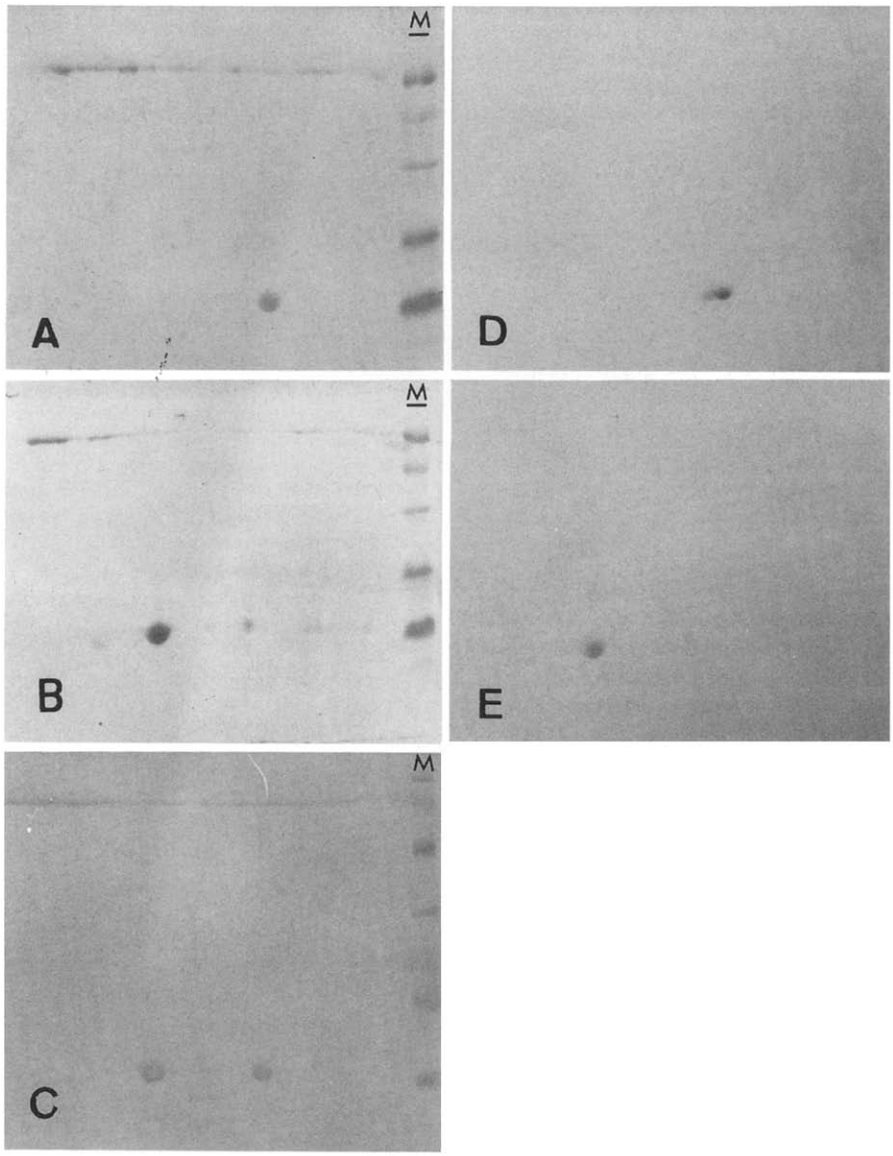

Fig. 1. Two-dimensional electrophoreses of HSP (A), cystatin-α (B) and mixture of these proteins (C), and immunoblotting analyses of HSP (D) and cystatin-α (E) using anti-HSP PoAb. (Lane M) Molecular mass markers (descending): phosphorylase *b* (94 kDa), bovine serum albumin (67 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa), soybean trypsin inhibitor (20.1 kDa) and lactalbumin (14.4 kDa).

bance was monitored at 280 or 410 nm. Activity of cathepsins B, H and L was assayed fluorometrically using aminomethylcoumarin substrates according to the method of Barrett and Kirschke [15]. Fluorescence was excited at 370 nm and monitored at 460 nm. The K_i value was determined by Dixon's method [16].

2.4. *Two-dimensional electrophoresis and immunoblotting analysis*

Two-dimensional electrophoresis for HSP and cystatin-α, and immunoblotting using PoAb against HSP were performed according to O'Farrell et al. [17] and Towbin et al. [18], respectively.

2.5. *Localization of HSP and cystatin-α*

The Lowicryl-embedded sections of newborn rat skin, previously fixed with 0.025% glutaraldehyde/2% paraformaldehyde, were prepared according to Roth et al. [19]. The in vivo location was demonstrated by immunofluorescent technique using PoAbs against HSP and cystatin-α.

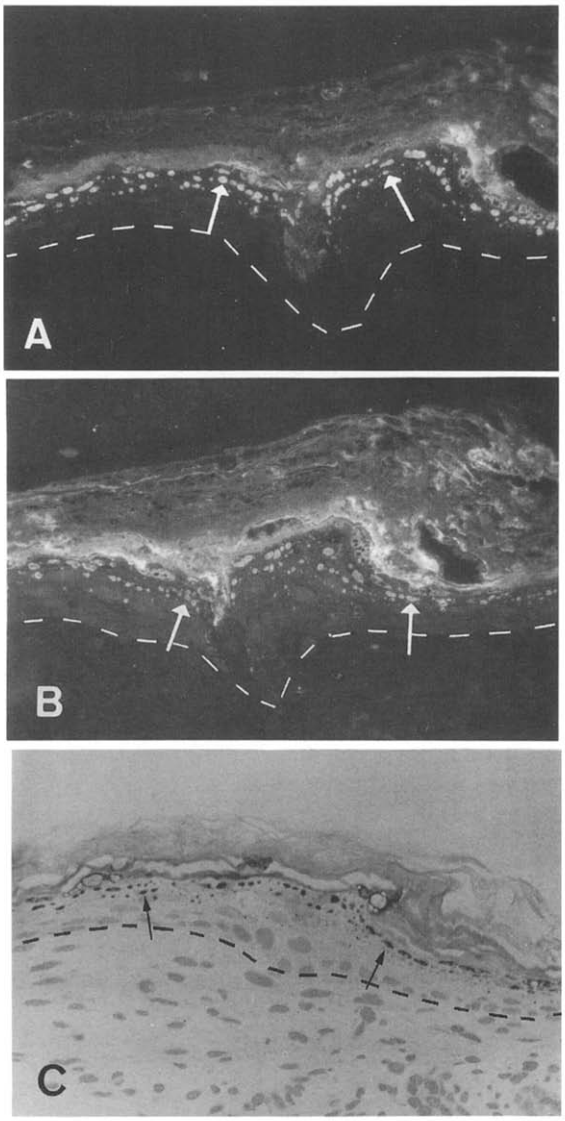

Fig. 2. In vivo location of HSP and cystatin-α by indirect immunofluorescence. Specific fluorescence was observed when the Lowicryl K4M-embedded section was reacted with anti-HSP PoAb (A) and anti-cystatin-α PoAb (B) and staining obtained with HE stain (C). A, B and C: × 120. Arrows indicate KHG. Dotted lines indicate epidermal-dermal junction.

Table III

Comparison of the inhibitory activities of HSP and cystatin-α for various proteinases

Enzyme	Substrate	ID_{50}[a]	
		HSP	Cystatin-α
Cysteine proteinases			
Papain	BAPNA	5.8 μg (K_i = 270 nM)	
	casein	14.6 μg	
Ficin	BAPNA	6.9 μg	
	casein	7.1 μg	
	Z-Phe-Arg-MCA	0.26 ng	0.17 ng
Cathepsin B	Z-Arg-Arg-MCA	23.5 ng	14.6 ng
Cathepsin H	Arg-MCA	0.6 ng	0.22 ng
Cathepsin L	Z-Phe-Arg-MCA	1.3 pg	7.7 pg
Serine proteinases			
Trypsin	BAPNA	>50.0 μg	
Chymotrypsin	Bz-Tyr-PNA	>50.0 μg	
Carboxy proteinase			
Pepsin	casein	>50.0 μg	
Metallo proteinase			
Thermolysin	casein	>50.0 μg	

[a] Amount necessary for 50% inhibition of activity

3. RESULTS

The inhibitory activity and the yields of HSP after each purification step are summarized in Table II. Purified HSP displayed a single protein spot on 2-dimensional electrophoresis (Fig. 1A). As shown in Fig. 1C, its isoelectric point was different from that of cystatin-α, though the molecular weights were quite similar. PoAb against HSP cross-reacted with cystatin-α (Fig. 1E). The location of HSP and cystatin-α using indirect immunofluorescent technique by anti-HSP and anti-cystatin-α PoAbs were identical as shown in Fig. 2A and B. And the profiles were well correlated with the distribution pattern of KHG in HE-stained specimen (Fig. 2C).

HSP inhibited only cysteine proteinases and not proteinases of other classes. The inhibitory activity of HSP against various proteinases is summarized in Table III, and compared with that of cystatin-α. Both HSP and cystatin-α inhibited the activities of various cysteine proteinases to a similar degree. The K_i value of HSP for papain was found to be 270 nM using BAPNA as the substrate. The inhibitory activity of HSP was rather stable to heat and extreme pHs.

4. DISCUSSION

Hematoxylin-stainable component protein of KHG was isolated and characterized as a cysteine proteinase inhibitor. Cysteine proteinase inhibitors have been reported to be located diffusely in the cytoplasm of the living cell layers, especially in the stratum spinosum or the stratum granulosum [3,20,21]. However, we found

HSP as well as cystatin-α to be located on KHG by indirect immunofluorescent techniques using anti-HSP and anti-cystatin-α PoAbs, respectively. As HSP is quite similar to cystatin-α in their molecular weights, amino acid compositions and inhibitory activities, HSP and recombinant cystatin-α [12] were examined by the immunoblotting technique using anti-HSP PoAbs. As both reacted with these PoAbs to the same extent, both should have many common epitopes in their molecules. The inhibitory activities of both HSP and cystatin-α against ficin were similar. Both had similar inhibitory activity against cysteine cathepsins increasing in the order cathepsin B, H and L. The amino acid compositions are quite similar to TPI(1) [7] and rat epidermal TPI [8]. Our preliminary observations show that the N-terminus of HSP is masked and the C-terminus of HSP is phenylalanine, being identical to that of cystatin-α, although complete amino acid sequence of HSP is not determined yet. On the basis of these data, it may be assumed that the amino acid sequence of HSP is quite similar to that of cystatin-α or is the same. In addition, it is possible to assume that HSP is phosphorylated as reported by Laber et al. in a similar manner as egg white cystatin [22].

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