Characterization of a new cysteine proteinase inhibitor of human saliva, cystatin SN, which is immunologically related to cystatin S

Satoko Isemura, Eiichi Saitoh and Kazuo Sanada

Department of Oral Biochemistry, Nippon Dental University, Niigata Faculty, Niigata 951, Japan

Received 7 January 1986; revised version received 27 January 1986

A new cysteine proteinase inhibitor, cystatin SN, was purified from human whole saliva by chromatography with DE32, Sephacryl S200, and CM-Sepharose CL6B. Cystatin SN is immunologically related to cystatin S and both inhibitors have a similar molecular mass of about 13 kDa. The new inhibitor, however, was clearly distinguished from cystatin S by its much higher p*I* value. These inhibitors showed similar inhibitory activity for ficin, but cystatin SN was a much better inhibitor for papain and dipeptidyl peptidase I. The amino acid sequence of cystatin SN deduced in the light of the known structure of cystatin S indicates that they have 10 different amino acid residues in the sequence comprising in total 113 residues.

Cysteine proteinase *Enzyme inhibitor* *Amino acid sequence* *(Human saliva)* *Cystatin S* *Cystatin SN*

1. INTRODUCTION

The structures of various cysteine proteinase inhibitors of low M_r from animals have been elucidated [1–9]. According to the structural homology, cystatin S characterized by us [1] belongs to the extracellular type [2–5] which can be distinguished from the intracellular type [6–9]. During the course of the study of this cysteine proteinase inhibitor of human saliva [1,10], we noticed the presence of several other proteins in saliva which cross-react immunologically with cystatin S. One such protein could be separated well due to the difference in net charge. Here, we describe the characterization of this new cysteine proteinase inhibitor which we term cystatin SN because of its immunological cross-reactivity with cystatin S and its p*I* value near neutrality.

2. MATERIALS AND METHODS

2.1. *Materials*

Cystatin S was prepared as in [1]. Briefly, a reconstituted solution from lyophilized human whole saliva was mixed with DE32 pre-equilibrated with 0.02 M Tris-HCl (pH 7.5). The mixture was poured onto a column and washed with the same buffer. The column was then eluted with a linear gradient of NaCl (0–2%) in the buffer. Cystatin S was isolated from fractions eluted at NaCl concentration higher than 1%. Preparation of rabbit anti-cystatin S antiserum, chemical reagents and enzymes used for sequence analysis and determination of K_i values were as in [1,10]. Carbobenzoxyphenylalanyl - arginyl - methylcoumarylamide (Z-Phe-Arg-MCA) was obtained from the Peptide Institute (Osaka), and glycyl-arginyl-methylcoumarylamide (Gly-Arg-MCA) from E.Y. Laboratories.

2.2. *Methods*

M_r values were estimated by SDS-polyacrylamide gel electrophoresis with a linear gradient of gel concentration from 10 to 20%. The isoelectric point was determined by isoelectric focusing on precoated polyacrylamide gel plates (Ampholine Pagplate, LKB) with a pH range of 3.5–9.5.

Published by Elsevier Science Publishers B.V. (Biomedical Division)
00145793/86/$3.50

2.3. *Enzyme assay*

Papain and ficin were assayed fluorimetrically with Z-Phe-Arg-MCA [11]. Dipeptidyl peptidase I was assayed as described by Gounaris et al. [12] except that Gly-Arg-MCA was used as substrate instead of Gly-Phe-MCA.

2.4. *Determination of protein and K_i values*

A sample was dissolved in water and the absorbance at 280 nm measured. Molar absorption coefficients were calculated from the protein content which was determined with a protein assay kit (Bio-Rad) using bovine serum albumin as standard and by assuming the molecular mass to be 13 kDa.

K_i values were obtained from Dixon plots [13].

2.5. *Isolation of cystatin SN*

Lyophilized, pooled whole saliva was reconstituted to the original volume and stirred overnight at 4°C [1]. Insoluble materials were removed by centrifugation. The supernatant was poured onto DE32 equilibrated with 0.02 M Tris-HCl (pH 7.5) in a glass filter. An unadsorbed fraction was collected and concentrated by ultrafiltration using YM 5 membranes (Amicon) and then applied onto a Sephacryl S200 column (1.7 × 130 cm) equilibrated with 0.01 M NH_4HCO_3. Fractions having both inhibitory activity and immunoreactivity with anti-cystatin S antiserum were lyophilized and dissolved in 0.02 M phosphate buffer (pH 6.8). The solution thus obtained was then applied onto a column of CM-Sepharose CL6B (fig.1).

2.6. *Peptide fragmentation and separation*

Reduced and carboxymethylated (Rcm) cystatin SN was digested with mouse submaxillary gland protease or with *Achromobacter* lysylendopeptidase as in [1]. Peptides precipitating from the reaction mixture after lysylendopeptidase digestion of Rcm-cystatin SN were collected by centrifugation and further digested with trypsin at 25°C for 6 h in 0.01 M NH_4HCO_3. Peptides obtained by these procedures were fractionated on a column of DEAE-Sepharose CL6B (0.7 × 24 cm) with linear gradient elution using 100 ml each of 0.01 M NH_4HCO_3 and 1 M NH_4HCO_3, and by reverse-phase high-performance liquid chromatography on a column of Synchropack RP8 (Synchrom) with acetonitrile in 5 mM phosphate buffer, pH 6.8.

2.7. *Amino acid sequence*

Amino acids were analyzed with an amino acid analyzer (Waters) and sequence analysis using manual Edman degradation was performed as in [1].

3. RESULTS

3.1. *Isolation of cystatin SN*

Cystatin SN was isolated from the fractions as indicated in fig.1. The preparation gave a single

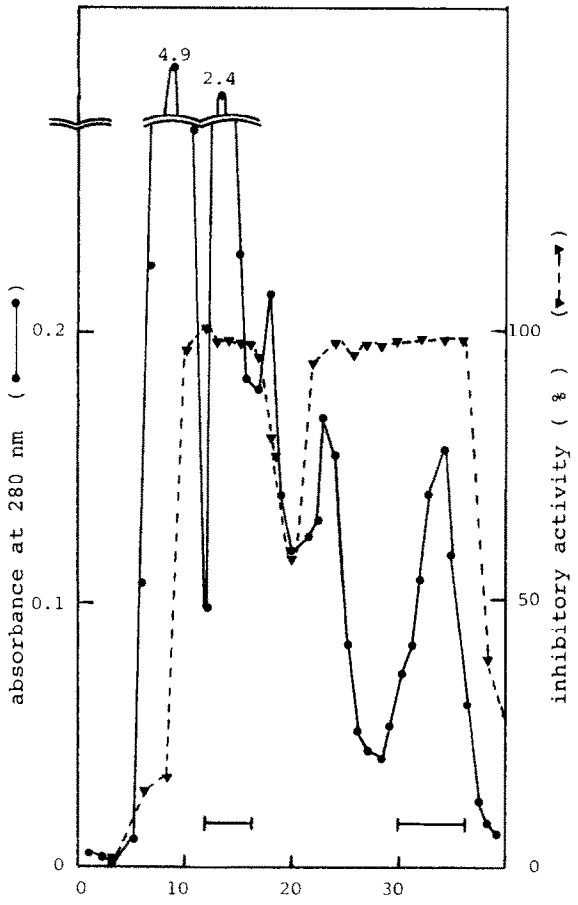

Fig.1. Purification of cystatin SN on a CM-Sepharose CL6B column (1.8 × 40 cm). A sample solution derived from a saliva fraction which was unadsorbed to DE32 was applied onto a column pre-equilibrated with 20 mM phosphate buffer (pH 6.8), and the column eluted with the same buffer. Fractions indicated by a bar showed reactivity with anti-cystatin S antiserum. Cystatin SN was obtained from frctions 32–36. Fractions were monitored for absorbance at 280 nm (●——●) and inhibitory activity (▼---▼) by using a 10 μl aliquot of each fraction and 23 ng ficin.

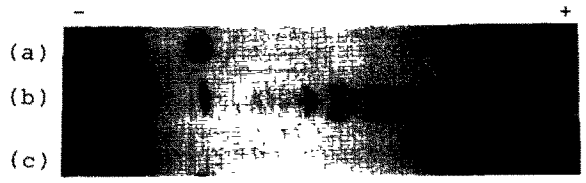

Fig.2. Electrophoretic analysis of cystatin SN in isoelectric focusing gel with a pH range of 3.5–9.5. (a) Cystatin SN, (b) unfractionated whole saliva, (c) cystatin S.

band on isoelectric focusing gel electrophoresis and was distinguished from cystatin S (fig.2). Table 1 lists the M_r estimated by SDS gel electrophoresis, isoelectric point, molar absorption coefficient and K_i values for ficin, papain, and dipeptidyl peptidase I in comparison with those of cystatin S.

Cystatin SN and cystatin S gave precipitin lines forming a spur with anti-cystatin S antiserum in the double immunodiffusion system, indicating their partial identity (fig.3). The final yield of cystatin SN was 4.4 mg from 1 l whole saliva.

3.2. *Amino acid sequence of cystatin SN*

The N-terminal 23 residues were determined with Rcm-cystatin SN (fig.4). Since a minor sequence (about 25%) starting with isoleucine and running one residue ahead of the main sequence could be followed after the second step and thereafter, it was suggested that this preparation contained a polypeptide lacking one of two isoleucine residues in the N-terminus. However, subsequent sequence studies with peptides derived by protease digestions (see below) suggested that cystatin SN was homogeneous except for the N-terminus.

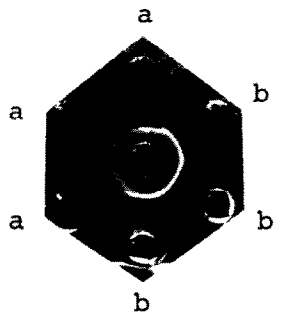

Fig.3. Double immunodiffusion analysis of cystatin SN. Rabbit anti-cystatin S serum (central well) was allowed to diffuse against cystatin SN (1.6 μg per well) in wells (a), and cystatin S (1.1 μg per well) in wells (b).

When Rcm-cystatin SN was digested with mouse submaxillary gland protease, peptides M1–M7 were obtained. Peptides M2–M4, M6 and M7 were completely sequenced, but peptides M1 and M5 only partially (fig.4).

When Rcm-cystatin SN was digested with lysylendopeptidase, peptides L1–L6 were obtained in a soluble form. The central part of the molecule (33–68) was obtained as a precipitate. Therefore, the precipitate was further digested with trypsin to yield soluble peptides LT1–LT6. These lysylendopeptidase peptides and lysylendopeptidase-trypsin peptides were sequenced as shown in fig.4. The determined amino acid sequences of Rcm-cystatin SN and its proteolytic fragments were sufficient to explain the whole molecule on the basis of the amino acid composition.

In view of the immunological cross-reactivity of cystatin SN with cystatin S (fig.3), one can expect that these proteins have highly homologous structures. Thus, in the light of the amino acid sequence of cystatin S [1], the proteolytic fragments of Rcm-

Table 1

Some characteristics of cystatin SN and cystatin S

	M_r	p*I*	$E^{1M}_{280nm,1cm}$ ($\times 10^4$)	K_i (nM)		
				Ficin	Papain	Dipeptidyl peptidase I
Cystatin SN	13000	7.5	1.8	4.4	21.4	26.3
Cystatin S	13000[a]	4.68	2.0	4.2	108.2	>630.2

[a] The calculated value from the determined sequence was 13268

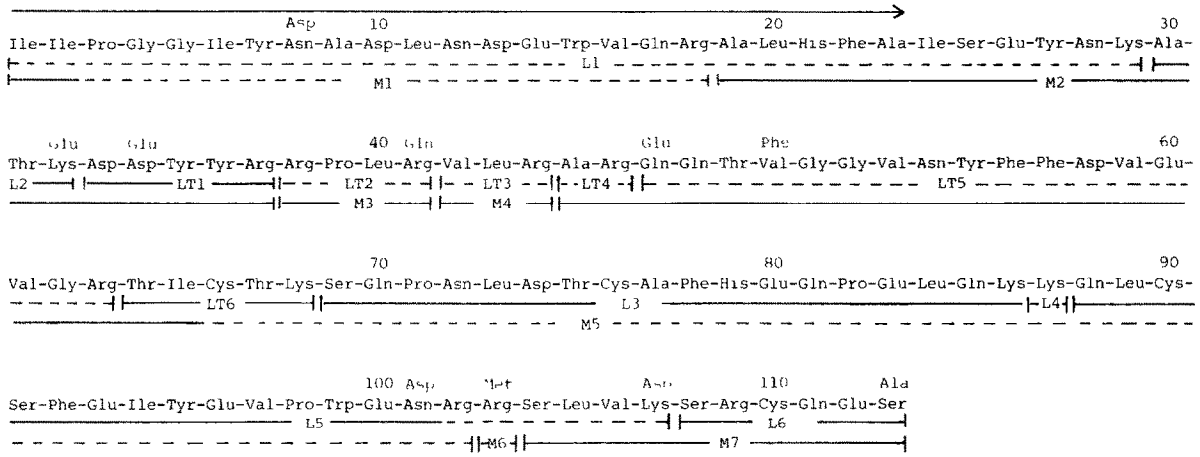

Fig.4. Amino acid composition and sequence of cystatin SN. Composition: Asp + Asn 12.5 (6 + 6), Thr 4.8 (5), Ser 5.6 (6), Glu + Gln 17.0 (9 + 8), Pro 5.6 (5), Gly 5.8 (5), Ala 6.8 (6), 1/2 Cys 3.4 (4), Val 7.5 (8), Ile 4.2 (6)*, Leu 7.5 (8), Tyr 4.8 (6), Phe 4.7 (5), His 1.9 (2), Trp – (2), Lys 6.0 (6), Arg 9.3 (10). Total 113 residues; M_r 13284; an asterisk indicates that a discrepancy between experimental and calculated values may be due to the incomplete hydrolysis of the Ile–Ile bond at the N-terminus. Numbers in parentheses are those from the composition determined after sequence analysis. The arrow above the sequence indicates the residues determined by Edman degradation of Rcm-cystatin SN. Bars beneath the sequence denote the fragments used for the sequence study which were obtained by cleavage with mouse submaxillary gland protease (M1–M7), lysylendopeptidase (L1–L6), and lysylendopeptidase plus trypsin (LT1–LT6). A solid part in a bar represents the sequenced part. The residues of cystatin S which differ from those of cystatin SN are shown above the corresponding residues of cystatin SN.

cystatin SN were placed in order as shown in fig.4. The amino acid compositions of the peptides which were isolated but not sequenced (fig.4) agreed with this structure completely.

4. DISCUSSION

Although cystatin SN was clearly separated from cystatin S by ion-exchange chromatography because of their difference in isoelectric point, these two saliva proteinase inhibitors showed immunological cross-reactivity which suggested their homologous structures. On the basis of this expected structural similarity and the determined amino acid sequences of polypeptides, a tentative amino acid sequence of cystatin SN was proposed as in fig.4. The structure indicates that cystatin SN has a sequence with 10 amino acid substitutions from that of cystatin S. These 10 residues introduce 9 positive net charges into cystatin SN, which explains the fact that the p*I* value of this inhibitor is much higher than that of cystatin S (table 1). In addition, these substitutions seem to be responsible for the stronger inhibitory activity for papain and dipeptidyl peptidase-I compared with cystatin S (table 1).

Our preliminary study has indicated that there is another inhibitor which is cross-reactive immunologically with cystatin S but has an amino acid sequence distinct from both cystatin S and cystatin SN. Therefore, human saliva appears to contain several cysteine proteinase inhibitors which are related to cystatin S but different from cystatin S in inhibitory specificity due to different structures.

REFERENCES

[1] Isemura, S., Saitoh, E. and Sanada, K. (1984) J. Biochem. 96, 489–498.
[2] Grubb, A. and Löfberg, H. (1982) Proc. Natl. Acad. Sci. USA 79, 3024–3027.
[3] Turk, V., Brzin, J., Longer, M., Ritonja, A. and Eropkin, M. (1983) Hoppe-Seyler's Z. Physiol. Chem. 364, 1487–1496.
[4] Schwabe, C., Anastasi, A., Crow, H., McDonald, J.K. and Barrett, A.J. (1984) Biochem. J. 217, 813–817.

[5] Hirado, M., Tsunasawa, S., Sakiyama, F., Niinobe, M. and Fujii, S. (1985) FEBS Lett. 186, 41–45.
[6] Takio, K., Kominami, E., Wakamatsu, N., Katsunuma, N. and Titani, K. (1983) Biochem. Biophys. Res. Commun. 115, 902–908.
[7] Machleidt, W., Borchart, U., Fritz, H., Brizn, J., Ritonja, A. and Turk, V. (1983) Hoppe-Seyler's Z. Physiol. Chem. 364, 1481–1486.
[8] Takio, K., Kominami, E., Bando, Y., Katsunuma, N. and Titani, K. (1984) Biochem. Biophys. Res. Commun. 121, 149–154.
[9] Ritonja, A., Machleidt, W. and Barrett, A.J. (1985) Biochem. Biophys. Res. Commun. 131, 1187–1192.
[10] Isemura, S., Saitoh, E., Ito, S., Isemura, M. and Sanada, K. (1984) J. Biochem. 96, 1311–1314.
[11] Barrett, A.J. (1980) Biochem. J. 187, 909–912.
[12] Gounaris, A.O., Brown, M.A. and Barrett, A.J. (1984) Biochem. J. 221, 445–452.
[13] Dixon, M. and Webb, E.C. (1979) in: Enzymes, 3rd edn, pp.332–380, Longmans, London.