Human Cystatin D

cDNA CLONING, CHARACTERIZATION OF THE ESCHERICHIA COLI EXPRESSED INHIBITOR, AND IDENTIFICATION OF THE NATIVE PROTEIN IN SALIVA*

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A cDNA coding for cystatin D, a human member of the cystatin protein family, has been cloned after specific amplification of reverse-transcribed parotid gland RNA. After replacing the segment encoding the putative 20-residue signal peptide with one encoding the Escherichia coli OmpA leader sequence, the cDNA was expressed in E. coli. The isolated recombinant protein exhibited $K_v$ values of 1.2 nM and $>1 \mu M$ for papain and cathepsin B, respectively. An antiserum raised against recombinant cystatin D recognized a protein in human saliva with electrophoretic mobility identical to that of the recombinant protein. Immunoenzymatic analysis revealed that this cysteine proteinase inhibitor is present in human saliva and tears at concentrations of 3.8 and 0.5 mg/liter, respectively, while it was not detected in seminal plasma, blood plasma, milk, or cerebrospinal fluid. Cystatin D purified from human saliva by immunosorption displayed a heterogeneous N-terminal end, with sequences starting at residues 5, 7, 9, and 11 of the predicted N-terminal portion of the mature protein. On the basis of structural and functional properties, cystatin D represents a novel cysteine proteinase inhibitor possibly playing a protective role against proteinases present in the oral cavity.

The cystatin superfamily comprises a group of proteinase inhibitors which are widely distributed in human tissues and body fluids, and which form tight and reversible complexes with cysteine proteinases such as cathepsins B, H, L, and S. The cystatins are most likely involved in the regulation of all cysteine proteinases in the body. The cystatins are present in body fluids, and which form tight and reversible complexes with cysteine proteinases such as cathepsins B, H, L, and S. The cystatins are most likely involved in the regulation of all cysteine proteinases in the body.

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Our recently cloned a new gene from a human genomic library, the structure of which is very similar to those of previously known family II cystatin genes (Freije et al., 1991). This gene contains coding information for a pre-protein of 142-amino acid residues, where the first 20 probably constitutes a signal peptide. The deduced polypeptide chain sequence of the mature protein, called cystatin D, displays regions homologous to the three regions forming the inhibitory center of other cystatins. Cystatin D would thus correspond to an as yet unidentified fifth human family II cystatin which, according to Northern blot studies (Freije et al., 1991), should be present in human saliva. In this work we report the cloning and expression in Escherichia coli of a CDNA coding for the putative cystatin D. We also show that the recombinant protein displays inhibitory activity against cysteine proteinases. Finally, we report the purification and characterization of cystatin D from human saliva.

EXPERIMENTAL PROCEDURES

Materials—Specimens of human parotid gland were obtained at autopsies performed within 15 h after death and frozen in liquid nitrogen and stored at -70 °C until used. The RNA PCR kit used for isolating RNA was produced by the alkaline lysis procedure and then digested with EcoRI. The resulting linearized plasmid, devoid of a NarI/EcoRI fragment downstream from the SmaI site of the cystatin C gene, was ligated into the plasmid pUC18 followed by the insertion of the cystatin C gene excluding the putative signal peptide. The resulting fragment containing the polynucleoty region of pUC18 followed by the transcription terminator from phage fd was isolated from pHd162 SP9b (Dalbåge et al., 1989a) and ligated to the above plasmid fragment, resulting in plasmid pHd388 in which the cystatin C gene is followed by the pUC18 polynucleoty and the fd transcription terminator. pHd388 was digested with EcoRI, the plasmid containing the Klenow fragment of EcoRI DNA polymerase, and then digested with Clal to remove the cystatin C gene including the OmpA signal peptide encoding fragment. A 100-bp Clal/PstI fragment containing the OmpA signal peptide encoding sequence was isolated from pHd22-2 (Dalbåge et al., 1989b) and ligated to the CDS fragment from pHd388, resulting in pHd389. The resulting construct thus contained the phage λ Cl temperature-sensitive repressor gene, the phage λ PR promoter, an optimized ribosome binding site, the OmpA signal peptide encoding sequence, a pUC18 polynucleoty, and the fd transcription terminator.

Expression of the Cystatin D Gene in E. coli—An additional oligonucleotide was synthesized which contained a recognition site for NarI (5'-GGCGGGCCGGAGTGCCTCG). This primer was used to amplify the coding sequence of cystatin D, excluding the putative signal sequence, using the oligonucleoty cyd-a (5'-GACAT- CCTCGCCCATGAC) and cysd-b (5'-GGTGGTCAGTGTGAC) as downstream primer and 25 ng of the plasmid pCysD1 as template. Cycling conditions were as above, except that only 30 cycles were carried out and the annealing temperature was 55 °C in the first 5 cycles and 60 °C for the remaining 25 cycles. The final product was digested with NarI and Stul and the resulting DNA fragment was ligated between the NarI and Smal sites of the vector pHd389. The resulting plasmid, called pCysD2-1, was transformed into E. coli strain MC1061 (Casadaban and Cohen, 1980).

Bacteria containing the expression plasmids pHd389 or pCysD2-1 were induced to produce recombinant protein as previously described for cystatin C production (Abrahamson et al., 1988; Dalbåge et al., 1989b). To extract the recombinant protein from the periplasm, the induced bacteria were collected by centrifugation and resuspended in one-twentieth of the original volume in 0.2 M Tris buffer, pH 9.0, with 20% sucrose and 0.1 M EDTA. After centrifugation, the bacteria were resuspended again in one-twentieth of the original volume in 10 mM Tris buffer, pH 9.0, by vigorous agitation for 10 min. Cell debris were removed by centrifugation at 20,000 × g for 15 min and the supernatant was stored at 4 °C until used.

Isolation of Recombinant Cystatin D—Periplasmic extracts were collected by 5-fold dilution of the recombinant bacteria in a Mono Q HR 5/5 column (Pharmacia LKB, Uppsala, Sweden) equilibrated in 20 mM ethanalamine buffer, pH 9.0. The elution was carried out with a gradient of the same buffer containing 1.0 M NaCl at a flow rate of 0.5 ml/min. Fractions containing recombinant cystatin D were pooled and gel chromatographed on a Superdex 75 HR 10/30 column (Pharmacia LKB) at a flow rate of 0.1 M ammonium acetate at a flow rate of 0.2 ml/min. Fractions of 0.2 ml were collected and those containing pure recombinant cystatin D, as judged by SDS-polyacrylamid gel electrophoresis, were pooled and lyophilized. All purification steps were performed at 4 °C.

Amino Acid Sequence Analysis—Isolated recombinant cystatin D (0.5 nmol) or protein bands blotted onto polyvinylidene difluoride membranes (Trans-Blot, Bio-Rad) were subjected to N-terminal sequence analysis by automatic Edman degradation using an Applied Biosystems 477A Sequencer in the presence of Polybrene (Hewick et al., 1981; Matsuura, 1987). The resulting phenylthiohydantoin derivatives were identified and quantitated using an on-line phenylthiohydantoin analyzer (model 120A) and the standard elution program of Applied Biosystems.

Determination of Cysteine Proteinase Inhibitory Activity—Concentrations of inhibitor active recombinant cystatin D in samples were determined by titration of the protein with Bz-Phe-Arg-NHMec as substrate in 100 μM sodium phosphate buffer (pH 7.5). The inhibition reaction was run at 0.5 nmol and protein bands blotted onto polyvinylidene difluoride membranes (Trans-Blot, Bio-Rad) were subjected to N-terminal sequence analysis by automatic Edman degradation using an Applied Biosystems 477A Sequencer in the presence of Polybrene (Hewick et al., 1981; Matsuura, 1987). The resulting phenylthiohydantoin derivatives were identified and quantitated using an on-line phenylthiohydantoin analyzer (model 120A) and the standard elution program of Applied Biosystems.
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before use. The enzyme concentrations in the assays were 0.05–0.25 nm. The highest cystatin D concentration tried in cathespin B assays was 100 nm. The inhibitor concentrations giving informative inhibition, i.e. resulting in a new steady state rate within 1 h after addition of inhibitor, were 20–50 nm in the papain assays. Substrate hydrolysis at 37 °C was monitored in a Perkin-Elmer Cetus LS50 fluorometer at excitation and emission wavelengths of 590 and 480 nm, respectively. Steady-state velocities before (v0) and after (v1) addition of inhibitor were found with the aid of linear regression using FLUSYS (Rawlings and Barrett, 1990a), a computer program provided by Neil Rawlings and Alan Barrett, Strangways Research Laboratory, Cambridge, MA. Apparent K values Kapp were calculated as the slope from the plot of [(v1-v0)/v0] versus K/v1 (Henderson, 1972). K values for hydrolysis of Z-Phe-Arg-NHMe under the assay conditions, of 80 µM for papain (Hall et al., 1992) and 250 µM for cathespin B (Kischke and Barrett, 1987), were used to compensate for the substrate-induced association of inhibitor, by the relationship: Kapp = K(1 + [S]/K).

Antiserum Production.—Polyclonal antisera against recombinant cystatin D were produced by injecting 0.2 mg of purified recombinant cystatin D emulsified in complete Freund’s adjuvant subcutaneously into each of two rabbits. Three weeks later, additional injections were given as a boost. Rabbits were bled 6 weeks after the first injection and every third week. The IgG fraction of the antiserum was isolated by chromatography on a protein A-Sepharose column (Pharmacia LKB).

Immunological Analyses—Proteins separated by SDS-PAGE (Laemmli, 1970) were electrophoretically transferred into nitrocellulose membranes (Bio-Rad, Bedford, MA) at 20 mA for 2 h in 25 mM Tris, 192 mM glycine, and 0.1% (v/v) methanol. After transfer, filters were blocked in phosphate-buffered saline with 10% bovine serum albumin for 30 min and then washed with phosphate-buffered saline containing 0.1% Tween 20. The rabbit antiserum against recombinant cystatin D was used for a 1:1000 dilution. After washing, the filters were incubated with [125I]-protein A (ICN Radiochemicals) for 30 min and then washed again with phosphate-buffered saline with 0.1% Tween 20. Immunoreactive bands were visualized by autoradiography using a Kodak X-Omat film and an exposure time of 8 h. Classical immunoelectrophoresis employing 1% (w/v) agarose gels in 75 mM barbital buffer, pH 8.6, was performed according to Scheidegger (1955).

The cystatin D concentration in different body fluids was measured by enzyme-amplified single radial immunodiffusion. (Löfberg and Grubh, 1979). Gels of 1% (w/v) agarose containing 10% (w/v) dextran T10 and 0.6% (v/v) of the rabbit antiserum against recombinant cystatin D were used. The precipitates were visualized by carbazole staining after incubation with horseradish peroxidase-labeled swine antibodies against rabbit immunoglobulins (Dako Inc., Copenhagen, Denmark). Recombinant cystatin D was isolated from the periplasmic extract of the bacteria E. coli MC1061 expressing the cDNA in MC1061 and the transformed bacteria were induced to produce the correct predicted lengths (data not shown).

The PCR product was subcloned into pEMBL19 and six independent clones were subjected to nucleotide sequence analysis. The nucleotide sequence of the cystatin D cDNA and the corresponding amino acid sequence are shown in Fig. 1. The nucleotide sequence of the cDNA obtained contained an open reading frame of 426 bp encoding 142 amino acids. This sequence differs by a single base (C instead of T at position 430) from the sequence described for the putative exon parts of the cystatin D gene (Freije et al., 1991). This difference at the DNA level results in an amino acid change (arginine specified by the cDNA sequence and cysteine by the genomic one). Hybridization studies with allele-specific oligonucleotides on a large number of genomic DNA samples have demonstrated that this change corresponds to a polymorphism in the population (Balbin et al., 1993).

Expression of Cystatin D in E. coli.—In order to elucidate whether the cloned cDNA sequence codes for a biologically active cysteine proteinase inhibitor, an attempt was made to express the cDNA in E. coli. To produce the putative mature protein in the periplasm of E. coli, the previously proposed signal peptide of cystatin D was replaced by the E. coli OmpA signal peptide. For this purpose, the sequence coding for the putative mature protein was amplified using a pair of oligonucleotides corresponding to the 3'- and 5'-ends of the sequence (Fig. 2). Due to the design of the 5'-primer (Cys-Nar), the amplified fragment contained the coding information for 2 additional amino acids (Ala-Pro) at the N-terminal end (Fig. 2).

The DNA fragment thus obtained was inserted in the polylinker region of the expression vector pH389. The resulting plasmid, called pCysD2–1, contained the coding sequence for the proposed mature cystatin D in-frame with the one coding for the signal peptide of OmpA.

The original vector (pHD389) as well as the recombinant plasmid (pCysD2–1) were transformed into E. coli MC1061 and the transformed bacteria were induced to produce the recombinant protein. The periplasmic contents were extracted by an osmotic shock procedure (Dalbge et al., 1989b) and the protein composition was analyzed by SDS-PAGE. As can be seen in Fig. 3, the periplasmic extract of the bacteria transformed with the recombinant plasmid contained a protein of about 17 kDa, which was not present in the control extracts. The yield of recombinant protein was approximately 100 mg/liter culture.

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Fig. 1. Human cystatin D cDNA. The sequence of the PCR-amplified cDNA is shown, with the amino acid sequence of the translation product below the nucleotide sequence. The residues which differ in the previously described genomic sequence are presented between brackets. The sequences of oligonucleotides cysd-a and cysd-b (complementary strand), used for the amplification, are underlined.

Fig. 2. Construction of the cystatin D expression plasmid. The differences in the 5′-oligonucleotide used to generate a fragment suitable for subcloning (Cys-Nar) with respect to the cystatin D sequence, as well as the differences in the translation product, are indicated.

To confirm that the 17-kDa polypeptide produced in E. coli was cystatin D, it was isolated from bacterial extracts using the strategy described under “Experimental Procedures.” Thus, extracts containing about 20 mg of total protein were chromatographed on a Mono Q column and the chromatogram obtained is shown in Fig. 4. As judged by SDS-PAGE, the putative cystatin D did not bind to the anion-exchange column, in contrast to most other proteins of the extract (Fig. 4, inset). The non-binding material was then fractionated by size-exclusion fast protein liquid chromatography and the eluate analyzed by 280-nm absorption and SDS-PAGE (Fig. 5). The main peak of the chromatogram corresponded to an isolated, single polypeptide chain protein, with a molecular mass of about 17 kDa (Fig. 5, inset). Automatic Edman degradation of the polypeptide chain identified a single sequence confirming the homogeneity of the protein preparation. The sequence obtained (Ala-Pro-Gly-Ser-Ala-Ser-Ala-Gln-Ser-Arg-Thr-Leu-Ala-Gly-Ile-His-Ala-Thr-Asp-Leu-Asn-Asp-Lys-Ser-Val-Gln-) corresponded exactly to the one deduced for cystatin D from the cDNA sequence with the exception of the 2 first residues (Ala-Pro) introduced in the recombinant cystatin D sequence as a result of the cloning procedure. In addition, the sequence obtained proved that the OmpA signal peptide had been properly processed and cleaved at the expected Ala-Ala bond during the secretion process. The slight differences between the molecular mass of cystatin D deduced from SDS-PAGE mobility (17 kDa), and that calculated from the amino acid sequence (about 14 kDa) are probably due to abnormal electrophoretical behavior of this low molecular mass protein, similar to what has been observed for cystatin C using the same buffer system (Abrahamson et al., 1988).

Inhibitory Activity of Recombinant Cystatin D—The cysteine proteinase inhibitory activity of recombinant cystatin D was investigated by attempts to determine the equilibrium constants for dissociation (K_i) for its interactions with the
model cysteine proteinases, papain, and cathepsin B. The cystatin D interaction with papain was slow, and steady-state enzyme activity rates after addition of inhibitor could only be readily observed at inhibitor concentrations $\geq 20$ nM. The inhibition obtained was compatible with a $K_i$ value for papain inhibition of 1.2 nM (S.E. = 0.09, $n = 4$). Significant inhibition of human cathepsin B activity could not be demonstrated even with a cystatin D concentration of 100 nM. Since a $K_i$ value of 1$\mu$M should have resulted in 30% reduction of enzyme activity under the conditions of the experiment, the $K_i$ value for cathepsin B inhibition was concluded to be $> 1$ $\mu$M.

Demonstration of Cystatin D in Human Saliva—A rabbit antiserum raised against isolated recombinant cystatin D was used in an attempt to demonstrate the presence of cystatin D in human saliva. The specificity of the antiserum was first tested by classical immunoelectrophoresis with human serum, purified preparations of the human cystatins A, B, C, S, SA, and SN, as well as recombinant cystatin D as antigens. Only recombinant cystatin D produced a precipitate. When fresh saliva was used as antigen in the procedure, a single immunoprecipitation arc was produced and in the same electrophoretic position as that produced by recombinant cystatin D. The proteins of fresh human saliva were also separated by SDS-PAGE, transferred to a nitrocellulose filter and tested for reactivity with the antiserum raised against recombinant cystatin D. Fig. 6 displays that human saliva contains a protein with virtually the same molecular mass and immunoactivity as recombinant cystatin D.

The antiserum against cystatin D was used to construct an enzyme-amplified single radial immunodiffusion procedure to allow quantitation of cystatin D in various body fluids. Ten samples each of saliva, tears, seminal plasma, and blood plasma, as well as four samples each of milk and cerebrospinal fluid, all from healthy individuals, were investigated. The cystatin D concentration in seminal plasma, blood plasma, milk, and cerebrospinal fluid was below the sensitivity limit of the procedure (0.1 mg/liter). The saliva samples had the highest cystatin D concentration (mean: 3.8 mg/liter, i.e. approximately 275 nM; range: 1.6–5.1 mg/liter). Tears displayed low, but detectable, cystatin D levels (mean: 0.5 mg/liter; range: <0.1–1.5 mg/liter).

Purification and Partial Characterization of Cystatin D from Saliva—In an attempt to isolate human cystatin D from saliva, the IgG fraction of the antiserum raised against recombinant cystatin D was coupled to a CNBr-activated Sepharose column. Fresh saliva containing a mixture of proteinase inhibitors was applied to the column. After washing of the column with proteinase-inhibitor-containing neutral buffer, the bound material was eluted with glycine buffer, pH 2.2, containing the same proteinase inhibitors. Immunoelectrophoresis was used to monitor the column effluent and revealed that only the acid effluent contained cystatin D immunoactivity. Agarose gel electrophoresis of the neutralized and concentrated acid effluent displayed two protein bands (A and B in Fig. 7). Band B had the same electrophoretic mobility as purified recombinant cystatin D and band A a slightly more anodal mobility. The proteins of a second aliquot of the concentrated effluent were separated by agarose gel electrophoresis, blotted onto polyvinylidene difluoride membranes, and subjected to automated Edman degradation. Two major sequences were obtained for the constituents of both band A and band B. All four agreed with segments of the N-terminal portion of the protein specified by the cystatin D cDNA (Fig. 8), starting at residues 5, 7, 9, and 11 of the predicted N-terminal portion of the mature protein (Freije et al., 1991).

**DISCUSSION**

The present work was undertaken after the cloning and characterization of a newly detected human gene (CST5).

The cystatin D gene was originally called CST4 when it was cloned and sequenced by Freije et al. (1991), but it has been renamed CST5 since the cystatin S gene was also called CST4 when it was simultaneously cloned (Saitoh et al., 1991). The designation CST5 for the human cystatin D gene will be officially used by the Human Gene Mapping Nomenclature Committee (P. McAlpine, personal communication).
seemingly coding for an additional member of the cystatin family of proteinase inhibitors, tentatively called cystatin D (Freije et al., 1991). Since no protein with the structural characteristics corresponding to this gene had been described, we tried to demonstrate that the protein encoded by the CST5 gene is a biologically active cysteine proteinase inhibitor. Our strategy was to isolate a cystatin D cDNA and then express it in a bacterial host. Since previous studies indicated that the CST5 gene is expressed in the parotid gland (Freije et al., 1991), the required cDNA was obtained by reverse transcription and PCR amplification of RNA from a sample of this tissue. Nucleotide sequence analysis of several cDNA clones confirmed the overall structural data derived from the sequence of the CST5 gene, including the location of all intron-exon junctions which had been proposed for the gene. Interestingly, comparison of the nucleotide sequence of the cystatin D cDNA with the previously determined genomic sequence revealed a single nucleotide difference resulting in a Cys/Arg variation in the amino acid sequence of the putative mature protein. The finding of both variants of the CST5 gene in the general population (Balbin et al., 1993) clearly indicates that this variation represents a common polymorphism and not cloning artifacts in the cDNA or genomic clones.

After characterization, the cystatin D cDNA was placed under the control of a heat inducible promoter and expressed in E. coli using a system very similar to the one previously used for the production of cystatin C with full biological activity (Abrahamson et al., 1988; Dalbøge et al., 1989b). The recombinant cystatin D was secreted to the periplasmic space indicating that the product had been properly directed to this bacterial compartment by the OmpA signal peptide fused to the putative N-terminal sequence of the mature protein. The recombinant protein was purified by a simple two-step chromatographic procedure which resulted in the isolation of large amounts of protein suitable for structural and functional analysis. The N-terminal sequence of the purified protein confirmed that the signal peptide had been efficiently processed at the expected position and that the protein product had the amino acid sequence predicted from the nucleotide sequence, including the 2 additional residues introduced in
the N-terminal end during the cloning process.

Functional analysis performed with the recombinant protein demonstrated that cystatin D is a functional cysteine proteinase inhibitor, although the equilibrium constant for dissociation of its complex with papain ($K_i = 1.2$ nM) was much higher than that of the cystatin C-papain complex ($K_i = 11$ mM; Lindahl et al., 1992). It is unlikely that this difference is due to the presence of 2 extra N-terminal residues (Ala-Pro) in recombinant cystatin D, since N-terminally extended Ala-Met-Glu-Ala-Glu-cystatin C displays virtually the same inhibitory properties as native cystatin C of human origin (Abrahamson et al., 1989). It is also noteworthy that recombinant cystatin D did not show any significant inhibitory activity against cathepsin B, suggesting that it may have a much more restricted inhibitory spectrum than other cystatins characterized to date (Barrett, 1987).

The availability of isolated recombinant cystatin D allowed the production of an antiserum suitable for testing the presence in human fluids of proteins related or identical to the recombinant protein. As anticipated from the expression of the CST5 gene in parotid gland (Freije et al., 1991), immunoblotting of saliva proteins separated by SDS-PAGE revealed the presence in human saliva of a protein with the expected size and immunoreactivity. It was possible to isolate the protein from saliva by the use of immunosorption but the isolated protein displayed a ragged N terminus, although maximal precautions were taken to prevent proteolytic degradation during the saliva collection and cystatin D isolation. A similar situation has been described for the isolation of cystatins S, SN, and SA from saliva (Hawke et al., 1987; Saitoh et al., 1988; Al-Hashimi et al., 1988; Isemura et al., 1991). The fact that the longest variant of cystatin D we could demonstrate in saliva is 4 residues shorter than the sequence predicted for the mature protein by using von Hejne’s algorithm (von Hejne, 1985; Freije et al., 1991) is probably due to proteolytic degradation, although it cannot be excluded that this cystatin D variant displays the authentic N-terminal sequence of the native protein. However, all sequence data obtained agree with the sequence for cystatin D deduced from the CST5 gene structure and therefore provide conclusive evidence that the cystatin D gene is normally expressed in at least some human tissues.

The quantitative analysis of the cystatin D concentration in several biological fluids showed a unique distribution for this cystatin, since it could only be demonstrated in saliva and tears but not in milk, seminal plasma, blood plasma, or cerebrospinal fluid. The mean saliva cystatin D concentration, approximately 275 nM, is slightly higher than the mean saliva level of cystatin C (Abrahamson et al., 1986). This concentration is sufficiently high to cause complete inhibition of papain in saliva, since our data show that the equilibrium constant for dissociation of cystatin D-papain complexes is more than 100 times lower (1.2 nM), which means that the enzyme-inhibitor equilibrium will be shifted almost totally towards the inhibitor form. This theoretical consideration indicates that cystatin D might have a physiological role in saliva as an inhibitor of cysteine proteinases with papain-like properties.

In this fluid, the inhibitor could play a protective role against the potentially harmful effects of proteinases of bacterial, fungal, viral, or cellular origin present in the oral cavity. Therefore, cystatin D may be considered as an additional component of the nonimmune protective system in this cavity, with an interesting parallelism to histatins, a family of histidine-rich proteins found in human parotid secretion and with fungistatic and antibacterial properties (Oppenheim et al., 1988; Sabatini et al., 1989). By contrast, the other known family II cystatins, including the so-called salivary cystatins S, SN, and SA, have a wider distribution in the body and may play different physiological roles in those biological fluids and secretions in which they are present.

The ability to obtain large amounts of functional recombinant cystatin D from engineered E. coli, as described here, will be helpful to further clarify the physiological role of this protein and its relationship to other cystatins, with special reference to the occurrence of distinctive specificities against the different cysteine proteinases. Finally, the availability of cDNA and genomic clones for cystatin D will facilitate comparative studies to elucidate the mechanisms involved in the differential expression of the genes encoding all members of this family of proteinase inhibitors.

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FIG. 8. Identified sequences for human salivary cystatin D. The sequences determined by automated Edman degradation of the two protein bands shown in Fig. 7 are given in the standard one-letter code.

Predicted mature cystatin D sequence

Band A proteins:

Band B proteins:

GSASQRTLAGGHATDLNDKS

TLAGG-HA

AGG-HATD

AQ-RTLAA

SRTLAGG

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