HUMAN TRYPSINOGENS IN THE PANCREAS AND IN CANCER

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Original publications

This thesis is based on the following articles which are referred to in the text by their Roman numerals:

I Itkonen O, Koivunen E, Hurme M, Alfthan H, Schröder T and Stenman UH. Time-resolved immunofluorometric assays for trypsinogen-1 and 2 in serum reveal preferential elevation of trypsinogen-2 in pancreatitis. J Lab Clin Med 115, 712-718 (1990).

II Koivunen E, Itkonen O, Halila H and Stenman UH. Cyst fluid of ovarian cancer patients contains high concentrations of trypsinogen-2. Cancer Res 50, 2375-2378 (1990).

III Itkonen O, Stenman UH, Osman S, Koivunen E, Halila H and Schröder T. Serum samples from pancreatectomized patients contain trypsinogen immunoreactivity. J Lab Clin Med 128, 98-102 (1996).

IV Itkonen O, Helin J, Saarinen J, Kalkkinen N, Ivanov KI, Stenman UH and Valmu L. Mass spectrometric detection of tyrosine sulfation in human pancreatic trypsinogens, but not in tumor-associated trypsinogen. FEBS J 275, 289-301 (2008).

Publication II was also included in the thesis entitled “Tumor-associated trypsinogen” by Ph.D, docent Erkki Koivunen, Department of Biological and Environmental Sciences, University of Helsinki, 1991.

Abbreviations

AA	amino acid
API	α_1-proteinase inhibitor
CCK	cholecystokinin
CF	cystic fibrosis
CTSB	cathepsin B
ECM	extracellular matrix
ESI	electrospray ionization
HP	hereditary pancreatitis
IRT	immunoreactive trypsinogen
LC	liquid chromatography
MAb	monoclonal antibody
MMP	matrix metalloproteinase
MS	mass spectrometry
MSMS	tandem mass spectrometry
PTM	post-translational modification
RIA	radioimmunoassay
RP	reverse-phase
TAP	trypsinogen activation peptide
TAT	tumor-associated trypsinogen
TATI	tumor-associated trypsin inhibitor
TIMP	tissue inhibitor of metalloproteinase
TPST	tyrosylprotein sulfotransferase
TR-IFMA	time-resolved immunofluorometric assay

Abstract

Human pancreatic juice contains two major trypsinogen isoenzymes called trypsinogen-1 and -2, or cationic and anionic trypsinogen, respectively. Trypsinogen isoenzymes are also expressed in various normal and malignant tissues. We aimed at developing monoclonal antibodies (MAbs) and time-resolved immunofluorometric assays recognizing human trypsinogen-1 and -2, respectively. Using these MAbs and assays we purified, characterized and quantitated trypsinogen isoenzymes in serum samples, ovarian cyst fluids and conditioned cell culture media.

In sera from healthy subjects and patients with extrapancreatic disease the concentration of trypsinogen-1 is higher than that of trypsinogen-2. However, in acute pancreatitis we found that the concentration of serum trypsinogen-2 is 50-fold higher than in controls, whereas the difference in trypsinogen-1 concentration is only 15-fold. This suggested that trypsinogen-2 could be used as a diagnostic marker for acute pancreatitis.

In human ovarian cyst fluids tumor-associated trypsinogen-2 (TAT-2) is the predominant isoenzyme. Most notably, in mucinous cyst fluids the levels of TAT-2 were higher in borderline and malignant than in benign cases. The increased levels in association with malignancy suggested that TAT could be involved in ovarian tumor dissemination and breakage of tissue barriers.

Serum samples from patients who had undergone pancreatoduodenectomy contained trypsinogen-2. Trypsinogen-1 was detected in only one of nine samples. These results suggested that the expression of trypsinogen is not restricted to the pancreas.

Determination of the isoenzyme pattern by ion exchange chromatography revealed isoelectric variants of trypsinogen isoenzymes in serum samples. Intact trypsinogen isoenzymes and tryptic and chymotryptic trypsinogen peptides were purified and characterized by mass spectrometry, Western blot analysis and N-terminal sequencing. The results showed that pancreatic trypsinogen-1 and -2 are sulfated at tyrosine 154 (Tyr154), whereas TAT-2 from a colon carcinoma cell line is not. Tyr154 is located within the primary substrate binding pocket of trypsin, thus Tyr154 sulfation is likely to influence substrate binding. The previously known differences in charge, substrate specificity and inhibitor binding between pancreatic and tumor-associated trypsinogens are suggested to be caused by sulfation of Tyr154 in pancreatic trypsinogens.

The terms “enzyme” (from the Greek *en* - in and *zume* - yeast) and “trypsin” were first suggested by a german scientist, Wilhelm (Willy) Friedrich Kühne (1837-1900), when he found a substance in bovine pancreatic juice that degraded other biological substances. He proposed the term “enzyme” for non-organized ferments and “trypsin” for the enzyme that breaks down proteins. Kühne presented this paper in the 4th February 1876 to the Heidelberger Naturhistorischen und Medizinische Verein, and was reprinted in 1976 (Kühne, 1976).

Trypsinogen was first characterized from cattle pancreatic extracts (Kunitz and Northrop, 1934, Northrop and Kunitz, 1932). In a study of Kunitz and Northrop (Kunitz and Northrop, 1935) bovine trypsinogen was shown to be activated either by enteropeptidase or active trypsin, indicating that the activation can be autocatalytic. Activation was shown to be pH dependent and maximal at pH 7.0 to 8.0. The molecular masses of purified trypsin, trypsin complexed with a polypeptide inhibitor, and polypeptide trypsin inhibitor were reported to be 36 500 Da, 40 000 Da and 6 000 Da, respectively. Trypsin was reversibly inhibited by the inhibitor. The activity, general properties and inhibition of various preparations of crystalline trypsin by the polypeptide inhibitor were also reported.

Before this study was started, tumor-associated trypsin inhibitor (TATI) had been isolated from urine of an ovarian cancer patient and shown to be identical to pancreatic secretory trypsin inhibitor (PSTI) (Huhtala et al., 1982). Elevated levels of TATI had been observed in urine from patients with ovarian, cervical and endometrial cancer. In search for a target protease for TATI, two trypsinogen isoenzymes were shown to be expressed in cyst fluid of mucinous ovarian tumors (Koivunen et al., 1989). The N-terminal amino acid sequences of these tumor-associated isoenzymes corresponded to those of pancreatic trypsinogen-1 and -2, respectively. However, the isoenzymes had different specificities for *p*-nitroanilide substrates, responded differently to various protease inhibitors and had different isoelectric points from those of trypsinogen-1 and -2. Therefore, they were named tumor-associated trypsinogen-1 and -2 (TAT-1 and TAT-2) (Koivunen et al., 1989).

Properties and biochemical characterization of human pancreatic trypsinogens

Trypsinogen (Enzyme Commission (EC) number 3.4.21.4) was first reported to occur in human pancreatic juice by Haverback et al. (Haverback et al., 1960) and was among the first human enzymes to be purified and characterized (Buck et al., 1962). Human pancreatic juice contains two major trypsinogen isoenzymes called trypsinogen-1 and -2, or cationic and anionic trypsinogen, respectively (Figarella et al., 1969, Keller and Allan, 1967, Rinderknecht and Geokas, 1972). Trypsinogen-2 is the most anionic protein in human pancreatic juice (Figarella et al., 1969). The proportion of trypsinogen-1 to trypsinogen-2 is about two to one in normal pancreatic juice (Figarella et al., 1969, Guy et al., 1978, Rinderknecht et al., 1979), and these two trypsinogens represent 19% of total proteins of pancreatic juice (Guy et al., 1978). Total trypsinogen concentration in human pancreatic juice is reported to be in the range of 4 to 40 µmol/L (Rinderknecht et al., 1979). A third, minor trypsinogen isoenzyme, called trypsinogen-3 or mesotrypsinogen, also occurs in human pancreatic juice (Rinderknecht et al., 1979, Rinderknecht et al., 1984, Scheele et al., 1981).

In some reports (Nyaruhucha et al., 1997, Scheele et al., 1981), but not in this thesis, the trypsinogen isoenzymes have been designated

Table 1. Properties of various human trypsinogens

Approved Gene Symbol[a]	Gene[b]	Isoenzyme	Synonym (not used in this study)	Predicted[c] MW (Da)	Predicted[c] pI	Predicted[d] charge at pH 7.0
PRSS1	T4	Trypsinogen-1	Cationic trypsinogen Trypsinogen-3[e]	24 184	7.6	1.3
		TAT-1			5 - 5.5[f]	
PRSS2	T8	Trypsinogen-2	Anionic trypsinogen Trypsinogen-1[e]	24 114	5.1	-6.7
		TAT-2		24 034[g]	4[f]	
PRSS3	T9	Trypsinogen-3	Mesotrypsinogen Trypsinogen-2[e] Trypsinogen-3 Isoform C	24 239	6.6	-0.6
		Trypsinogen-4	Trypsinogen-3 Isoform A Trypsinogen-3 Isoform B Brain trypsinogen	24 239 24 239	6.6 6.6	
	T1 pseudogene	Trypsin X3				
	T2 pseudogene					
	T3 pseudogene					
	T5 pseudogene	Trypsinogen B				
	T6 transcribed pseudogene	Trypsinogen C		24 125	6.6	-0.4
	T7 pseudogene	Trypsinogen D				

[a]The Human Genome Organization, HUGO 2005, [b]Rowen et al. 1996, [c]UniProtKB/Swiss-Prot, with sulfation of Tyr154 in trypsin-1 and -2, respectively, [d]Roach et al. 1997, [e]Scheele et al. 1981, [f]Koivunen et al. 1989, [g](IV)

according to their isoelectric point, which causes confusion about the nomenclature of trypsinogens. Table 1 summarizes the properties and nomenclature of human trypsinogens and genes used throughout this study. UniProtKB/SwissProt numbering of amino acid residues (http://au.expasy.org/) is used unless otherwise stated.

Human trypsin-1 is inhibited completely by the Kunitz bovine inhibitor (BPTI), strongly by lima bean trypsin inhibitor (LBTI), moderately by the Bowman-Birk inhibitor and porcine Kazal inhibitor (PSTI) and only weakly by soybean trypsin inhibitor (SBTI). Chicken ovomucoid shows no inhibition whatsoever. Trypsin-2 is totally inhibited by BPTI, strongly by SBTI and LBTI, considerably more strongly inhibited than trypsin-1 by the Bowman-Birk inhibitor and porcine PSTI, and weakly by ovomucoid. Furthermore, human inter-α-trypsin inhibitor (ITI) inhibits trypsin-2 more readily than trypsin-1. Human α_1-proteinase inhibitor (API) completely inhibits both trypsin-1 and -2 (Figarella et al., 1975, Mallory and Travis, 1975). The optimal activity of trypsin isoenzymes is between 7.5 and 8.5 (Rinderknecht et al., 1984) and the proteolytic activities of trypsin-1 and -2 have been found to be identical (Colomb et al., 1978). Generally, trypsin-2 is characterized to be less stable and undergo faster autolysis than trypsinogen-1 and it is more sensitive to inhibition by naturally occuring proteinase inhibitors (Colomb et al., 1978, Mallory and Travis, 1973, Rinderknecht and Geokas, 1972).

Trypsinogen-3 occurs at very low concentrations and represents probably <0.5% of the proteins and <5% of trypsinogens in normal human pancreatic juice (Nyaruhucha et al., 1997, Rinderknecht et al., 1984). Trypsinogen-3 resembles trypsinogen-1 and -2 in many properties, but it is not inhibited by either human pancreatic secretory trypsin inhibitor (PSTI) or other naturally occuring trypsin inhibitors (Nyaruhucha et al., 1997, Rinderknecht et al., 1984, Sahin-Tóth, 2005). In contrast, active trypsin-3 rapidly hydrolyzes and degrades the Kunitz-type trypsin inhibitor SBTI and Kazal-type inhibitor PSTI (Szmola et al., 2003). Furthermore, trypsin-3 was shown to selectively and rapidly cleave the Lys10-Thr11 peptide bond of API. Subsequent mutagenesis studies revealed that trypsin-3 exhibits an unusually restricted S' subsite specificity but can efficiently digest Lys/Arg – Ser/Thr peptide bonds in polypeptide substrates (Szepessy and Sahin-Tóth, 2006).

The stability of trypsin-3 resembles that of trypsin-2, its pH optimum is at 8.2 and it needs calcium for full enzymatic activity (Rinderknecht et al., 1984). In contrast to the conserved features of trypsinogen isoenzymes in various species, an arginine instead of glycine is present at residue 198 in trypsinogen-3 (Roach et al., 1997). This residue was shown by x-ray chrystallography (Katona et al., 2002) to be located in the substrate binding pocket of trypsinogen and was suggested to be the structural basis for the nearly total resistance of trypsin-3 to natural trypsin inhibitors (Nyaruhucha et al., 1997). This was confirmed by studies on trypsinogen-3 mutant Arg198Gly (Szmola et al., 2003). Paradoxally, the Arg198 substitution also renders trypsin-3 more resistant to autocatalytic degradation (Szmola et al., 2003).

Pancreatic trypsinogen-1, -2 and -3 cDNAs contain 741 bp of coding region, which translates to a single polypeptide chain with 247 amino acids (AA) (Table 2). The three preproenzymes share about 87% homology and all the typical sequence features of a trypsinogen: a fifteen AA signal sequence, an eight AA activation peptide, the catalytic triad comprising residues His63, Asp107 and Ser200, the four key pocket specificity residues Asp194, Gln197, Gly217 and Gly227, and the six cysteine residues needed for the conserved disulfide bridges (48-64, 171-185, and 196-220). In addition to these, trypsinogen-2 contains a disulfide bridge at 30-160 and trypsinogen-1 and -3 contain two additional ones at 30-160 and 139-206. Interestingly, all other known trypsinogens from higher vertebrates contain six disulfide

Table 2. Amino acid sequences of transcribed human trypsinogens. The AA numbering below is in accordance with the ATG initiator codon of trypsinogen-1 as 1. Signal peptide and activation peptide of the isoenzymes is in italics and underlined, respectively. The conserved catalytic triad is in bold, and Tyr154 that is sulfated in pancreatic trypsinogen-1 (TRY1) and -2 (TRY2), respectively, is shaded. The ID numbers and sequences are according to UniProt and *Nemeth et al., 2007, and the gene products of PRSS3 are designated TRY4 for brain isoforms A and B, and TRY3 for the pancreatic isoform C. TRG C is trypsinogen C.

```
                                                                                 1         10                   20
P07477|TRY1                 ---------- ---------- ---------- ---------- ---------M NPLL-ILTFV AA-------- --ALAAPFDD
P07478|TRY2                 ---------- ---------- ---------- ---------- ---------M NLLL-ILTFV AA-------- --AVAAPFDD
P35030|TRY4 ISOFORM A       ---MCGPDDR CPARWPGPGR AVKCGKGLAA ARPGRVERGG AQRGGAGLEL HPLLGGRTWR AARDADGCEA LGTVAVPFDD
P35030|TRY4 ISOFORM B*      ---------- ---------- ---------- ---------- -------LEL HPLLGGRTWR AARDADGCEA LGTVAVPFDD
P35030|TRY3 ISOFORM C       ---------- ---------- ---------- ---------- ---------M NPFL-ILAFV GA-------- --AVAVPFDD
Q8NHM4|TRG C                ---------- ---------- ---------- ---------- ---------M NPLL-ILAFV GA-------- --AVAVPFDD

                                    30         40         50         60         70         80         90        100
P07477|TRY1                 DDKIVGGYNC EENSVPYQVS LNSGYHFCGG SLINEQWVVS AGHCYKSRIQ VRLGEHNIEV LEGNEQFINA AKIIRHPQYD
P07478|TRY2                 DDKIVGGYIC EENSVPYQVS LNSGYHFCGG SLISEQWVVS AGHCYKSRIQ VRLGEHNIEV LEGNEQFINA AKIIRHPKYN
P35030|TRY4 ISOFORM A       DDKIVGGYTC EENSLPYQVS LNSGSHFCGG SLISEQWVVS AAHCYKTRIQ VRLGEHNIKV LEGNEQFINA AKIIRHPKYN
P35030|TRY4 ISOFORM B*      DDKIVGGYTC EENSLPYQVS LNSGSHFCGG SLISEQWVVS AAHCYKTRIQ VRLGEHNIKV LEGNEQFINA AKIIRHPKYN
P35030|TRY3 ISOFORM C       DDKIVGGYTC EENSLPYQVS LNSGSHFCGG SLISEQWVVS AAHCYKTRIQ VRLGEHNIKV LEGNEQFINA AKIIRHPKYN
Q8NHM4|TRG C                DDKIVGGYTC EENSVPYQVS LNSGSHFCGG SLISEQWVVS AGHCYKPHIQ VRLGEHNIEV LEGNEQFINA AKIIRHPKYN

                                   110        120        130        140        150        160        170        180
P07477|TRY1                 RKTLNNDIML IKLSSRAVIN ARVSTISLPT APPATGTKCL ISGWGNTASS GADYPDELQC LDAPVLSQAK CEASYPGKIT
P07478|TRY2                 SRTLDNDILL IKLSSPAVIN SRVSAISLPT APPAAGTESL ISGWGNTLSS GADYPDELQC LDAPVLSQAE CEASYPGKIT
P35030|TRY4 ISOFORM A       RDTLDNDIML IKLSSPAVIN ARVSTISLPT APPAAGTECL ISGWGNTLSF GADYPDELKC LDAPVLTQAE CKASYPGKIT
P35030|TRY4 ISOFORM B*      RDTLDNDIML IKLSSPAVIN ARVSTISLPT APPAAGTECL ISGWGNTLSF GADYPDELKC LDAPVLTQAE CKASYPGKIT
P35030|TRY3 ISOFORM C       RDTLDNDIML IKLSSPAVIN ARVSTISLPT APPAAGTECL ISGWGNTLSF GADYPDELKC LDAPVLTQAE CKASYPGKIT
Q8NHM4|TRG C                RITLNNDIML IKLSTPAVIN AHVSTISLPT APPAAGTECL ISGWGNTLSS GADYPDELQC LDAPVLTQAK CKASYPLKIT

                                   190        200        210        220        230        240    247
P07477|TRY1                 SNMFCVGFLE GGKDSCQGDS GGPVVCNGQL QGVVSWGDGC AQKNKPGVYT KVYNYVKWIK NTIAANS
P07478|TRY2                 NNMFCVGFLE GGKDSCQGDS GGPVVSNGEL QGIVSWGYGC AQKNRPGVYT KVYNYVDWIK DTIAANS
P35030|TRY4 ISOFORM A       NSMFCVGFLE GGKDSCQRDS GGPVVCNGQL QGVVSWGHGC AWKNRPGVYT KVYNYVDWIK DTIAANS
P35030|TRY4 ISOFORM B*      NSMFCVGFLE GGKDSCQRDS GGPVVCNGQL QGVVSWGHGC AWKNRPGVYT KVYNYVDWIK DTIAANS
P35030|TRY3 ISOFORM C       NSMFCVGFLE GGKDSCQRDS GGPVVCNGQL QGVVSWGHGC AWKNRPGVYT KVYNYVDWIK DTIAANS
Q8NHM4|TRG C                SKMFCVGFLE GGKDSCQGDS GGPVVCNGQL QGIVSWGYGC AQKRRPGVYT KVYNYVDWIK DTIAANS
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bonds (Kenesi et al., 2003). The activation peptide contains a cluster of anionic residues, namely four aspartates preceding a positively charged lysine residue, a conserved feature of mammalian trypsinogens (Chen and Feréc, 2000c, Roach et al., 1997).

Human trypsinogen-1 and trypsin-1 may occur in single- and double-chain forms. Single-chain trypsin is called β-trypsin. The double-chain form is produced by autocatalytic cleavage of the Arg122-Val123 peptide bond of β-trypsin, the two chains being held together by a disulfide bond. Studies using recombinant human trypsinogen-1 reveal these two forms to be functionally identical. However, cleavage of the Arg122-Val123 bond in trypsinogen-1 inhibits trypsinogen-1 autoactivation and this may be one of the protective mechanisms of premature trypsinogen activation in the pancreas (Kukor et al., 2002b).

Both trypsinogen-1 and -2 contain two calcium binding sites (Abita et al., 1969, Colomb and Figarella, 1979). The so called high affinity calcium ion binding loop (Glu75 – Glu85) common to trypsinogen and active trypsin maintains the enzyme in its active form and simultaneously protects it from autodegradation (Abita et al., 1969, Bode and Schwager, 1975a, Bode and Schwager, 1975b, Szmola and Sahin-Tóth, 2007). The second calcium binding site located in the region of four Asp residues of the activation peptide is present in the zymogen only. The balance between trypsin activation and degradation is regulated by Ca^{2+} concentration. At low Ca^{2+} concentrations chymotrypsin C (denoted enzyme Y by Rinderknecht (Rinderknecht et al., 1988)) cleaves with high selectivity the Leu81-Glu82 bond within the Ca^{2+} binding loop of trypsin-1 resulting in rapid degradation and loss of trypsin activity by subsequent tryptic cleavage of the autolysis site Arg122-Val123. Increasing the Ca^{2+} concentration progressively inhibits the degradation of trypsin-1 by chymotrypsin C. At 1 mmol/L Ca^{2+} chymotrypsin C mediated cleavage of the Leu81-Glu82 is essentially completely inhibited by stabilizing the Ca^{2+} binding loop. Thus, at the high Ca^{2+} concentration in the duodenum chymotrypsinogen C facilitates trypsinogen autoactivation (see below), whereas at low Ca^{2+} concentration in the lower small intestines chymotrypsin C promotes trypsin degradation (Szmola and Sahin-Tóth, 2007).

Detailed analysis of the evolution of trypsinogen activation peptide demonstrated that the Asp-Asp-Asp-Asp-Lys sequence in mammalian trypsinogens has evolved to inhibit autoactivation and enhance cleavage by enteropeptidase (Chen et al., 2003a). Under physiological conditions in the pancreatic juice (pH 8 and 1 mmol/L Ca^{2+}) this calcium site is probably saturated in trypsinogen-1 but not in trypsinogen-2. This facilitates trypsinogen-1 autoactivation. The Ca^{2+} binding site common to trypsin and trypsinogen has been reported to have pKa (Ca^{2+}) values of 2.8 and 3.4 and the Ca^{2+} binding site present in trypsinogen only has pKa(Ca^{2+}) values of 3.3 and 2.7 for trypsinogen-1 and -2, respectively (Colomb and Figarella, 1979).

Extrapancreatic trypsinogen expression

Extrapancreatic expression of human trypsin immunoreactivity or mRNA has been detected in the Paneth cells of the gastrointestinal mucosa (Bohe et al., 1986, Ghosh et al., 2002), in the brain (Wiegand et al., 1993), male genital tract (Paju et al., 2000), epithelial cells of the skin, esophagus, stomach, small intestine, lung, kidney, liver, and extrahepatic bile duct, and splenic macrophages, monocytes and lymphocytes, the nerve cells of hippocampus and cerebral cortex in the brain (Kawano et al., 1997, Koshikawa et al., 1998), colonic mucosa (Cottrell et al., 2004), vascular endothelial cells (Koshikawa et al., 1997), cerebrospinal fluid (Critchley et al., 2000), synovial cells and synovial fluid (Stenman et al., 2005), tracheal aspirate fluid and lung tissue (Cederqvist et al., 2003), in human bronchoalveolar lavage fluid (Prikk et al., 2001), and in human milk (Borulf et al., 1987).

Koivunen et al. showed that two trypsinogen isoenzymes are expressed in cyst fluid of mucinous ovarian tumors (Koivunen et al., 1989). The N-terminal amino acid sequences of these tumor-associated isoenzymes corresponded to those of pancreatic trypsinogen-1 and -2, respectively. However, the isoenzymes had different specificities for *p*-nitroanilide substrates, responded differently to various protease inhibitors and had isoelectric points different from those of pancreatic trypsinogen-1 and -2. Therefore, they were named tumor-associated trypsinogen-1 and trypsinogen-2 (TAT-1 and TAT-2) (Koivunen et al., 1989). TAT-1 and TAT-2 were found to be less anionic than trypsinogen-1 and -2 when separated by ion exchange chromatography (Koivunen et al., 1991b). However, the nucleotide sequence of TAT-2 and pancreatic trypsin-2 is identical (Sorsa et al., 1997). Since then, trypsinogen immunoreactivity or mRNA has been detected in various cancers like stomach, pancreas, ovary, lung, bladder, esophagus, bile duct, and colon cancers, and carcinoma cell lines (Bernard-Perrone et al., 1998, Bjartell et al., 2005, Hirahara et al., 1995, Hotakainen et al., 2006, Kato et al., 1998, Kawano et al., 1997, Kawano et al., 1997, Koivunen et al., 1991b, Koshikawa et al., 1992, Koshikawa et al., 1994, Miyagi et al., 1995, Miyata et al., 1999, Nyberg et al., 2002, Ohta et al., 1998, Oyama et al., 2000, Paju et al., 2004, Stenman et al., 2003, Terada et al., 1995, Terada et al., 1997, Williams et al., 2001, Yamashita et al., 2003).

Tumor-associated trypsins as well as other proteinases have been recognized as significant factors in cancer progression and metastatic processes such as cellular invasion, degradation of extra-cellular matrix proteins, angiogenesis and tissue remodeling as reviewed in (Nyberg et al., 2006). Extracellular proteolysis in cancer can be initiated by the urokinase plasminogen activator (uPA), uPA receptor (uPAR) and plasminogen, which in turn activates latent matrix metalloproteinases (MMPs). MMPs are secreted or transmembrane proteins that are capable of digesting extracellular matrix (ECM) and basement membrane components under physiological conditions. MMPs are associated with metastatic phenotype of malignant cells and they are considered to be the major functional contributors to metastatic processes (Chambers and Matrisian, 1997). Trypsin, too, degrades many ECM components (Koivunen et al., 1991a, Koshikawa et al., 1992, Moilanen et al., 2003, Stenman et al., 2005) but it is also a potent activator of several MMPs (Imai et al., 1995, Koivunen et al., 1989, Moilanen et al., 2003, Nyberg et al., 2002, Paju et al., 2001b, Sorsa et al., 1997, Umenishi et al., 1990) and could thus initiate proteinase cascades and participate in modulation of tumor cell behavior.

Tumor-associated trypsin expression has been shown to correlate with malignancy in various cancers and of the four known trypsin isoforms, TAT-2 seems to be most common in tumors (Hirahara et al., 1998, Ichikawa et al., 2000, Kato et al., 1998, Miyata et al., 1999, Nyberg et al., 2002, Paju et al., 2004, Yamamoto et al., 2001, Yamamoto et al., 2003). On the other hand, in microarray analysis trypsinogen IVb and trypsinogen C (see below) gene expression have been shown to be up-regulated in non-small cell lung cancer metastasis (Diederichs et al., 2004).

Trypsinogen genes

Trypsinogens are encoded by the protease, serine (*PRSS*) genes. Eight trypsinogen genes (denoted T1 to T8) divided into two clusters, have been located within the β T-cell receptor (TCR) locus on chromosome region 7q35 (Rowen et al., 1996). Of these, five (T4 to T8) are tandemly arrayed 10-kb locus-specific repeats at the 3' prime end of the β TCR locus. These repeats exhibit 90 to 91% overall nucleotide similarity, and embedded within each is a trypsinogen gene. Each gene contains five exons that span approximately 3.6 kb. In addition, there are two pseudo trypsinogen genes and one relic trypsinogen gene at the 5' prime end of the β TCR locus (T1 to T3), all in inverted transcriptional orientation. Earlier, T4 and T8, also known as *PRSS1* and *PRSS2*, have been identified as the cDNAs for

trypsinogen-1 and -2, respectively (Emi et al., 1986) (Table 1).

Polymerase chain reaction (PCR) analyses of pancreas, thymus and liver suggest that the third apparently functional trypsinogen gene (T6) in the β TCR locus may be expressed in minute amounts in the thymus (Rowen et al., 1996). This is further supported by expressed sequence tags (ESTs) AA295419 and AA295738. Comparison of the three-dimensional structures of T4, T6 and T8 gene products suggests that the catalytic triad of His63, Asp107 and Ser200 and the nature of substrate binding pocket are highly conserved. However, the protein product of T6 might interact differentially with other proteins, as suggested by variations in surface charge and shape distributions (Chen and Feréc, 2000c, Rowen et al., 1996). T6 may represent a transition state between a functioning duplicated gene and a nonfunctional pseudogene (Chen et al., 2001). The protein product of T6 (trypsinogen C) has so far not been identified.

The T1 gene was described to be a pseudogene (Rowen et al., 1996), but it could be a protein-coding gene according to Entrez database used by the National Center for Biotechnology Information (www.ncbi.nlm.nih.gov). The protein product of T1 gene (trypsin X3) has not yet been identified. T2 and T3, T5 and T7 have been identified as nonfunctional pseudogenes (Rowen et al., 1996).

A third trypsinogen cDNA and its product has independently been identified as trypsinogen-3 and -4 (Nyaruhucha et al., 1997, Tani et al., 1990, Wiegand et al., 1993). The chromosomal location of the gene encoding this isoenzyme was located to chromosome region 9p13 (Rowen et al., 1996). This gene (T9 or *PRSS3*) is formed by segmental duplications originating from chromosomes 7q35 and 11q24 and it has two distinct promoters derived from each of the originating chromosomes. Thus, the transcripts of *PRSS3* display two variants of exon 1 and share exons 2 to 5 which encode the active protease (Rowen et al., 2005).

The gene coding for functional trypsinogen-3 spans about 3.6 kb, it is duplicatively transferred from chromosome 7 and expressed in the pancreas (isoform C). The *PRSS3* variant coding for brain trypsinogen-4 spans 48.6 kb and it is a hybrid of an exon copied from chromosome 11 and four exons copied from chromosome 7. Allelic variants of this splice form (isoform A) are called a and b. Another splice form named isoform B includes an additional exon derived from chromosome 7 after the chromosome 11 derived exon. Translation initiation site of the isoform A a/b splice forms is thought to be in the first exon derived from chromosome 11 starting from AUG translation initiation codon and coding for a 72-residue leader peptide (Nemeth et al., 2007). Translation of the B isoform is predicted to start in its second exon coding for a 28-residue leader peptide. This leader peptide has leucine as an initiator amino acid, and it is encoded starting from a CUG initiation codon (Nemeth et al., 2007).

The leading exon determines the pathway of the protein. In the pancreas, a secretory pancreatic trypsinogen-3 is produced. However, a cytosolic trypsinogen-4 missing the typical leader peptide of secreted proteins is produced in the brain (Wiegand et al., 1993), epithelial cells of the colon, prostate, lung (Cottrell et al., 2004), several cancer cell lines, uterus, heart, hypothalamus and cerebellar cortex (Rowen et al., 2005). On the other hand, trypsinogen-4 a/b contains four Arg-X-X-Arg furin cleavage-recognition sites (Molloy et al., 1992) and trypsinogen-4 (a-form) has been detected in vesicles in transfected cell lines and in epithelial cells, supporting the possibility of an alternative secretion pathway (Cottrell et al., 2004, Wiegand et al., 1993).

No traces of trypsinogen-4 isoform A were found in the human brain by sequencing trypsinogen-4 samples isolated from human brain following a short post mortem delay (Nemeth et al., 2007). Instead, only isoform B of trypsinogen-4 was identified. When trypsinogen-4 is expressed in the U87 human glioblastoma cell line, the relative expression

level of isoform A using an AUG initiation codon was elevated as compared to the expression level of isoform B with the CUG initiation codon. These results suggest that CUG as the initiation codon may control the expression level of the protein. Thus, directing the incorporation of N-terminal leucine rather than methionine into isoform B of trypsinogen-4 is suggested to keep trypsinogen-4 expression in the brain at a relatively low level (Nemeth et al., 2007). The AA sequences of human trypsinogen-1, -2, -3 isoform C, -4 isoform A, -4 isoform B and trypsinogen C are presented in Table 2.

Regulation of pancreatic trypsinogen gene expression and secretion

Regulation of gene expression

The PTF1 complex. The exocrine pancreas is formed by acinar cells that synthesize and secrete digestive enzymes. Pancreas-specific expression of the about twenty acinar secretory enzymes at very high levels is controlled largely by the pancreatic transcription factor 1 (PTF1) complex (Cockell et al., 1989). PTF1 is an unusual heterotrimeric transcription factor. It contains a class A basic helix-loop-helix (bHLH) protein (p75), which is required for import of the transcription factor into the nucleus (Sommer et al., 1991) and two DNA-binding subunits previously called p48 and p64 (Roux et al., 1989). The P48/PTF1a subunit is an exocrine pancreas-specific bHLH protein (Krapp et al., 1996) that is unable to bind to DNA alone and has to oligomerize with p64 in order to do so (Krapp et al., 1996, Sommer et al., 1991). P64 has been shown to be mammalian suppressor of hairless (RBP-J) or its paraloque, RBP-L. In the adult pancreas, RBP-L provides the strong transcriptional activity of the PTF1 complex that drives the high-level expression of the digestive enzyme genes (Beres et al., 2006).

PTF1 binds to DNA in the 5' promoter region of acinar digestive enzyme genes (Cockell et al., 1989, Rose et al., 2001). The binding sites of the PTF1 complex are bipartite with an E-box (preferably CACCTG) and a TC-box (TTTCCCA) spaced one or two helical turns apart, center to center (Cockell et al., 1989, Rose et al., 2001). Whereas an E-box is sufficient to bind the P48-bHLH heterodimer and a TC-box is sufficient to bind the P48-RBP heterodimer, the trimeric complex requires both binding sites. Moreover, the binding of the trimeric complex is highly cooperative and can be much greater than the sum of the individual bindings, i.e. E-box for the bHLH and the TC-box for the RBP. This means that the formation of the trimeric PTF1 complex creates a synergistic dependence on the presence of both DNA sites spaced appropriately (Beres et al., 2006).

Cholecystokinin. It is known that acinar cell growth, energy production, gene expression and protein synthesis are also regulated by secretagogues. Cholecystokinin (CCK) increases the synthesis of pancreatic proteases including trypsinogen-1 by a prolonged effect on mRNA levels in the rat (Rosewicz et al., 1989). The binding of CCK to its cell surface receptor activates the mitogen-activated protein kinase (MAPK) cascades by various pathways like the extracellular signal-regulated kinases 1 and 2 (ERK1/2) cascade, the Jun N-terminal kinase (JNK) cascade and the p38 MAPK cascade, resulting in activation of gene transcription, protein translation, metabolism and functions of the cytoskeleton (Duan et al., 1995, Williams, 2001). CCK also activates the phosphoinositide 3-kinase – mammalian target of rapamycin – 70-kDa ribosomal protein S6 kinase (PI3K – mTOR - $P70^{S6K}$) signalling pathway, that primarily regulates protein synthesis at mRNA level, but is also required for mitogenesis (Crozier et al., 2006, Williams, 2001). Furthermore, using rat pancreatic acinar cells, CCK has been shown to activate the transcription factor nuclear factor-κB (NF-κB), which is required for the production of chemokines and cytokines by pancreatic acinar cells (Han et al., 2001). Thus, CCK also affects acinar cell gene transcription, protein synthesis and growth in several ways.

Dietary components. Dietary components can regulate digestive enzyme transcription, as with each meal the pancreas must synthesize new digestive enzymes to replace those secreted. In humans, enteral but not parenteral (intravenous) feeding has been demonstrated to increase trypsinogen synthesis (O'Keefe et al., 2006) and serum trypsinogen-1 immunoreactivity transiently (Florholmen et al., 1984a). Furthermore, dietary amino acids, especially branched chain AAs, have been shown to regulate pancreatic protein synthesis at the translation/initiation level, independently of hormonal and neuronal input, by phosphorylation of eukaryotic initiation factor eIF4E, its binding protein 4E-BP1, the ribosomal protein S6 kinase, and the formation of the eIF4F complex (Sans et al., 2006).

Other factors. Chemically modified tetracyclines (CMTs) and doxycycline (DOXY), which are chemical inhibitors of MMPs, have been shown to down-regulate the expression of TAT-2 mRNA and TAT-2 secretion by human COLO-205 cells (Lukkonen et al., 2000). Utilizing cDNA approach to identify genes differentially regulated during pancreatic regeneration after partial pancreatectomy in mice, the mitogenic Reg3β protein was shown to be induced in the acinar pancreas. Under these conditions, there was a 1.53-fold change in trypsin-2 gene expression (De Leon et al., 2006).

Regulation of secretion

The newly synthesized zymogens are segregated into condensing vacuoles, which undergo maturation to zymogen granules and are stored in the apical pole of the acinar cell. The secretion of pancreatic digestive enzymes is controlled physiologically by the vagal nerve, whose postganglionic neurons release acetylcholine, and by gastrointestinal hormones such as CCK, serotonin (Owyang and Logsdon, 2004), secretin, vasoactive intestinal polypeptide (VIP) and neuromedin C (Williams, 2001). Meal-stimulated CCK release from the intestinal mucosa represents the major physiological pathway for trypsinogen secretion (Owyang, 1996). The action of acetylcholine and CCK is mediated by G-protein coupled receptors on acinar cells (Williams, 2001). Human acinar cells contain mostly CCK2 receptors and proteinase-activated receptor-2 (PAR-2). Also CCK1 receptors have been detected in human pancreatic acinar cells (Galindo et al., 2005). CCK2 receptors bind both CCK and gastrin with high affinity (Owyang and Logsdon, 2004) whereas PAR-2 is activated by trypsin (Nguyen et al., 1999). It has also been suggested, that CCK stimulation of human pancreatic cells is regulated by an indirect mechanism of stimulation of afferent neurons (Ji et al., 2001). The neurohormonal regulation of pancreatic exocrine secretion is reviewed in (Nathan and Liddle, 2002).

Intracellular Ca^{2+} is considered to be the primary signaling factor in acinar cells as it triggers the fusion of zymogen granules with the apical plasma membrane and exocytosis. Levels of intracellular Ca^{2+} are modulated by activated G proteins and other signalling molecules like phospholipase C β, inositol triphosphate (IP_3), diacylglycerol, cyclic ADP ribose, nicotinic acid adenine dinucleotide phosphate, and by intracellular and plasma membrane Ca^{2+} ATPase pumps, and plasma membrane Ca^{2+} channels (Petersen, 2004, Turvey et al., 2005, Williams, 2006). The endoplasmic reticulum has been established as the primary site for Ca^{2+} release, but the acidic lysosomal-like compartment and mitochondria are also involved (Williams, 2006). The packaging, movement, and fusion of zymogen granules to the apical membrane of acinar cells is affected by several zymogen granule membrane proteins like the SNARE proteins, small G proteins of the Rab family, cyclic AMP, diacylglycerol, and actin filaments (Wasle and Edwardson, 2002, Williams, 2006).

Activation of trypsinogen to trypsin

Activation by enteropeptidase

The intrinsic catalytic activity of trypsinogen is ~10^8-fold lower than that of trypsin (Pasternak et al., 1998). Trypsinogens are activated into trypsins on cleavage of the eight-AA activation peptide by enteropeptidase (EC 3.4.21.9, also known as enterokinase) when they enter the duodenum. Enteropeptidase is a membrane-bound serine protease located in the enterocytes and goblet cells of the brush-border and glycocalyx of the duodenum and the proximal 15 cm of jejunum (Hermon-Taylor et al., 1977, Imamura and Kitamoto, 2003). It is an N-glycosylated, disulfide-linked heterodimer that is derived from a single-chain precursor. The heavy chain anchors enteropeptidase in the intestinal brush border membrane and the light chain is the catalytic subunit, which has the same mechanism of action as trypsin and chymotrypsin (Kitamoto et al., 1994, Light and Janska, 1989). Enteropeptidase itself has been proposed to be activated by a trypsin- and chymotrypsin-like protease called duodenase that cleaves a Lys-Ile bond in the amino terminus of the enteropeptidase light chain (Zamolodchikova et al., 2000).

The specificity of enteropeptidase for cleavage after Lys has been proposed to be consistent with the presence of Asp981 at the base and two Gly residues at the sides of the specificity pocket that binds the P1 substrate residue. The Arg-Arg-Arg-Lys sequence at residues 886 to 889 may interact directly with the Asp residues in positions P2 to P5 of trypsinogen substrates (Kitamoto et al., 1994). However, it has been shown by site-directed mutagenesis of human trypsinogen-1 that the four Asp residues in the activation peptide are not required for enteropeptidase recognition and they confer only a modest catalytic improvement of enteropeptidase-mediated trypsinogen activation in humans (Nemoda and Sahin-Tóth, 2005). Human trypsinogen-1 and -2 are activated by enterokinase at the same rate (Colomb and Figarella, 1979).

Autoactivation

Trypsinogen-1 and -2 can also be autoactivated by either human trypsin at the same rate, but the affinity of both trypsin-1 and -2 is higher for trypsinogen-1 than for trypsinogen-2. In presence of 1 mmol/L calcium at pH 5.6 the autoactivation of trypsinogen-1 becomes predominant compared to enterokinase activation. This suggests that under physiological conditions in the duodenum, enteropeptidase is the starter of trypsinogen activation but the predominant subsequent mechanism becomes trypsinogen autoactivation (Colomb and Figarella, 1979, Nemoda and Sahin-Tóth, 2005). Contrarily to trypsinogen-1 and -2, pancreatic trypsinogen-3 can neither autoactivate nor activate or degrade other pancreatic zymogens (Sahin-Tóth, 2005, Szilagyi et al., 2001, Szmola et al., 2003).

Activation by cathepsin B

Trypsinogen can be activated by lysosomal cysteine protease cathepsin B (CTSB) *in vitro* (Figarella et al., 1988) and *in vivo* in a mouse model (Halangk et al., 2000). There are several reports to support this activation mechanism in humans, too. Cathepsin B is abundantly present also in the human pancreatic secretory compartment and it is secreted together with trypsinogen into pancreatic juice (Kukor et al., 2002a). CTSB activates human trypsinogen-1 with trypsin yield of about 30% of that produced by enterokinase *in vitro* (Lindkvist et al., 2006). CTSB has been shown to activate recombinant trypsinogen-3 more readily than trypsinogen-1 or -2 at pH 4.0 (Szmola et al., 2003). This suggests that the premature intracellular activation of trypsinogen in acute pancreatitis might be initiated by the action of CTSB on trypsinogen-3 leading to degradation of PSTI, which contributes to the development of human pancreatitis. Furthermore, the Lys26Val mutation in the CTSB propeptide region is associated with tropical calcific pancreatitis (TCP) (Mahurkar et al., 2006). This mutation could affect CTSB

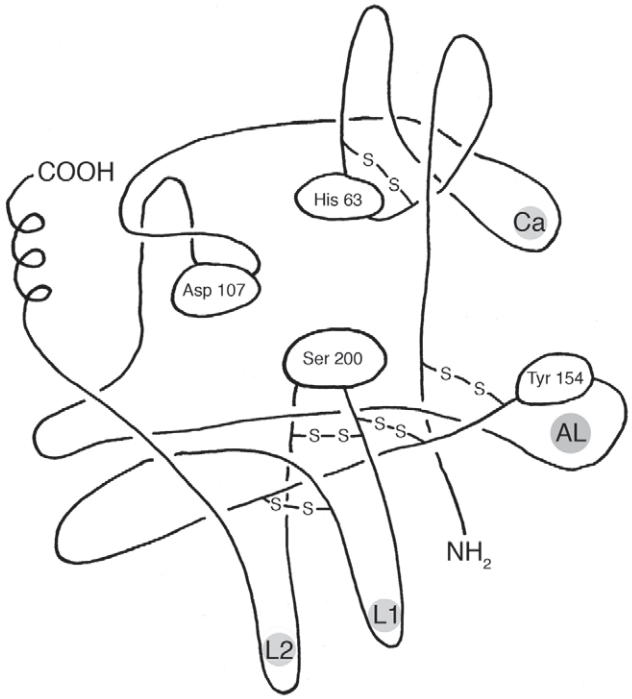


Figure 1. A shematic diagram of human trypsinogen-1. His63, Asp107 and Ser200 form the catalytic triad. L1 and L2, concerved loops which control the specificity of trypsin; AL, autolysis loop; Ca, calcium binding loop; -S-S-, disulfide bond.

trafficing and sorting to a lysosome or a zymogen granule.

Premature and intracellular activation of trypsinogen in experimental pancreatitis has been shown to depend on the presence of CTSB in CTSB deficient mice (Halangk et al., 2000), isolated rat pancreatic acini (Halangk et al., 2002, Saluja et al., 1997) and pancreatic homogenates from mice and rats (Van Acker et al., 2002). Asn29Ile mutation in trypsinogen-1 is associated with HP (Gorry et al., 1997). As compared to native human trypsinogen-1, the activation rate of recombinant Asn29Ile trypsinogen-1 by CTSB is increased threefold even in the presence of PSTI. This suggests that activation of trypsinogen by CTSB may play a role in the development of human pancreatitis (Szilagyi et al., 2001). However, there are several findings incompatible with the so called cathepsin B hypothesis (Klonowski-Stumpe et al., 1998, Lerch et al., 1993, Teich et al., 2002, Tooze et al., 1991), so the physiological and the pathophysiological role of CTBS as trypsinogen activator remains speculative.

Factors affecting trypsinogen activation

pH and calcium concentration. Autoactivation of recombinant human trypsinogens has been shown to be pH- and calcium-dependent *in vitro* (Kukor et al., 2003). Acidic pH stimulates autoactivation of recombinant human trypsinogen-1, but inhibits that of trypsinogen-2. At pH 8 in the presence of calcium at low concentration (<1 mmol/L) trypsinogen-2 exhibits minimal autoactivation due to rapid zymogen degradation, whereas trypsinogen-1 autoactivation is stimulated in a calcium concentration-dependent manner. Increasing

the calcium concentration progressively inhibits trypsinogen-1 autoactivation. A similar effect on trypsinogen-1 can be seen at high ionic strength (100 mmol/L NaCl). In contrast, calcium at 5 mmol/L stimulates autoactivation and inhibits autodegradation of trypsinogen-2. The effect of high NaCl concentrations on trypsinogen-2 was less significant.

During CCK hyperstimulation, the apical pole of isolated mouse acinar cells undergo a Ca^{2+} -dependent change characterized by local trypsin activation and replacement of the normal zymogen granules by vacuoles (Raraty et al., 2000). Sustained increase in cytosolic Ca^{2+} concentration has been demonstrated by an ion exchange mechanism to increase the free Ca^{2+} concentration and decrease the pH in the zymogen granules of mouse acinar cells resulting in premature activation and stabilization of trypsin (Yang et al., 2007).

The N-terminal sequence of four Asp residues has a negative effect on the hydrolysis of the Lys-Ile bond by trypsin as studied by using bovine and porcine trypsin (Abita et al., 1969). However, the slow hydrolysis of the Lys-Ile bond by trypsin is accelerated by calcium binding to the Asp residues of the activation peptide by decreasing the K_m of the reaction, i.e. improving the binding of trypsinogen to trypsin. This effect is mediated by neutralizing the high concentration of negative charges of the activation peptide (Abita et al., 1969, Nemoda and Sahin-Tóth, 2005).

Chymotrypsin C. The trypsinogens liberate the eight AA activation peptide Ala-Pro-Phe-Asp-Asp-Asp-Asp-Lys upon activation. In addition, a pentapeptide Asp-Asp-Asp-Asp-Lys is also formed from trypsinogen-1 (Guy et al., 1978, Nemoda and Sahin-Tóth, 2006). Chymotrypsin C (or caldecrin) specifically cleaves the Phe18-Asp19 peptide bond in the trypsinogen activation peptide removing the N-terminal tripeptide (Nemoda and Sahin-Tóth, 2006). Autoactivation of this N-terminally truncated trypsinogen-1 is stimulated 3-fold. This effect is dependent on the presence of Asp218, which forms part of the S3 subsite on trypsin. The N-terminal truncation of trypsinogen-1 is presumed to result in a conformational change within the remainder of the activation peptide, which repositions Asp21 and thereby mitigates the Asp21-Asp218 electrostatic repulsion (Nemoda and Sahin-Tóth, 2006). As chymotrypsinogen C is activated by trypsin, this reaction establishes a novel positive feedback mechanism in the digestive enzyme cascade of humans. The hereditary pancreatitis (HP) -associated mutation Ala16Val in trypsinogen-1 increases the rate of chymotrypsin C processing of the activation peptide four-fold and causes accelerated trypsinogen-1 activation *in vitro*. Chymotrypsin C also cleaves off the N-terminal tripeptide from human trypsinogen-2, but it has no significant effect on the autoactivation trypsinogen-2, which contains Tyr in place of Asp218 (Nemoda and Sahin-Tóth, 2006). The corresponding residue in trypsinogen-3 is His218.

Sulfation. As shown by us (IV) and others (Sahin-Tóth et al., 2006, Scheele et al., 1981), pancreatic trypsinogens are sulfated at Tyr154, which together with His46 lines the S'2 binding site (Gaboriaud et al., 1996, Schellenberger et al., 1994). This negatively charged modification has been proposed to modify interactions between trypsin and various substrates and inhibitors (Gaboriaud et al., 1996, Szilagyi et al., 2001). Even though the catalytic activity of sulfated pancreatic and non-sulfated recombinant trypsin-1 are essentially identical, the autoactivation of sulfated pancreatic trypsinogen-1 is 1.4-fold faster in the presence of 1 mmol/L Ca^{2+} and 2.4-fold faster in the presence of in 10 mmol/L Ca^{2+} than that of the non-sulfated recombinant form. In contrast, autoactivation of trypsinogen-2 is unaffected by Tyr154 sulfation (Sahin-Tóth et al., 2006).

Structural features. There are several structural features in the trypsinogen molecule that regulate its activation. The stability of the zymogen and the slow hydrolysis of the Lys23-Ile24 bond seem to be important mechanisms of protection against accidental

activation (Abita et al., 1969). Biochemical characterization of pancreatitis-associated activation peptide mutations in human trypsinogen-1 confirmed the importance of Asp residues in the activation peptide for control of autoactivation (Chen et al., 2003a, Teich et al., 2000). Suppression of autoactivation by electrostatic repulsion between the Asp residues in the activation peptide and the surface of trypsinogen-1 (Asp218 in the S3-S4 subsite) is further supported by site-directed mutagenesis in recombinant human trypsinogens (Chen et al., 2003a, Nemoda and Sahin-Tóth, 2005). Moreover, trypsin has up to eleven-fold preference for Arg over Lys at the P1 position of peptide substrates (Craik et al., 1985, Hedstrom et al., 1996), so use of Lys instead of Arg at the scissile bond also protects against autoactivation. The neutralizing effect of the high concentration of negative charge at the activation peptide by calcium binding provides yet another regulatory element (Nemoda and Sahin-Tóth, 2005).

Trypsinogen and trypsin structure and mechanism of catalysis

Structure

The crystal structures of human pancreatic trypsin-1 (Gaboriaud et al., 1996), brain trypsin-4 (Katona et al., 2002), trypsin and trypsinogen from bovine (Bode et al., 1976, Fehlhammer et al., 1977, Finer-Moore et al., 1992, Huber et al., 1974, Kossiakoff et al., 1977, Stroud et al., 1974) and from other species (Huang et al., 1994, Perona et al., 1993) have revealed highly conserved structural motifs within the trypsin family, the structural basis of substrate specificity as well as the mechanism of catalysis. Structurally, the trypsinogen molecule consist of two six-stranded beta barrels and the active site cleft is located between the two barrels (Figure 1).

The trypsinogen molecule consists of different functional domains; catalytic, substrate recognition and zymogen activation domains. However, the functional processes are not separate. More detailed domains that can be characterized within the mentioned ones are the activation peptide (Ala16 to Lys23), the calcium binding loop (Glu75 to Glu85), autolysis loop (Gly145 to Asp156) and oxyanion hole (backbone NHs of Gly198 to Ser200). In addition, up to 200 water molecules both inside the trypsin(ogen) molecule and on its surface are important in serving hydrogen bonds to stabilize the three-dimensional structure of trypsinogen and trypsin and to participate in the catalytic reaction. Internal water clusters are well conserved in various trypsin(ogen)s, and they are frequently shaped as water channels forming extensive hydrogen-bonding networks linked to the protein backbone (Bartunik et al., 1989, Finer-Moore et al., 1992, Krem and Di Cera, 1998, McDowell and Kossiakoff, 1995).

The eight AA activation peptide of trypsinogens and Asp199 stabilize the inactive trypsinogen conformation (Pasternak et al., 1998). Approximately 85% of the structures of trypsinogen and trypsin are identical (Fehlhammer et al., 1977, Kossiakoff et al., 1977) and trypsinogen (and chymotrypsinogen) has weak intrinsic activity towards small active site titrants (Kerr et al., 1975). The oxyanion hole, which is important for stabilization of the tetrahedral intermediate in the catalysis, and the primary binding site of trypsinogen are deformed, which renders the zymogen inactive. Upon cleavage of the activation peptide, the α-amino group of the new N-terminal Ile24 folds into a pocket and forms a buried salt bridge between the carboxylate group of Asp199 (Robinson et al., 1973), a mechanism coined “molecular sexuality” (Bode and Huber, 1976). However, hydrophobic interactions of the Ile24 side-chain provide the more stabilization energy for the trypsinogen to trypsin conversion that the salt bridge (Hedstrom et al., 1996). The resulting 170° rotation of the Asp199 side chain triggers a conformational change in the S1 binding site and oxyanion hole, which produces active enzyme (Fehlhammer et al., 1977). The Ile-Val dipeptide, analogous to the N-terminus of active trypsin, can also cause activation of trypsinogen without

cleavage of the activation peptide. This data indicates that zymogen activation is based on a conformational change (Bode and Huber, 1976).

Substrate binding

The substrate recognition sites include the polypeptide binding site, the binding pockets for the side-chains of the peptide substrate and surface loops outside the substrate binding pocket (Hedstrom et al., 1992). The nomenclature of the binding sites is based to that proposed by Schechter and Berger, where P1-P1' denotes peptide residues on the acyl and leaving group side of the scissile bond, respectively (Schechter and Berger, 1967). The adjacent peptide residues are numbered outward, and the S1, S1' etc. denote the corresponding enzyme binding sites.

The primary substrate-binding pocket. The disulfide bond Cys196 – Cys220, and the segments between Asp194 – Asp199, Ser215 – Cys220 and Pro226 – Tyr229 form the primary substrate-binding pocket called S1 binding site in active trypsin (Fehlhammer et al., 1977, Varallyay et al., 1997). The substrate specificity towards peptide bonds following Arg or Lys is mainly defined by three conserved residues; Asp194 at the bottom of the substrate binding pocket and Gly217 and Gly227 residues, which together create the negatively charged S1 site (Huber et al., 1974, Perona et al., 1995). The S1-P1 interaction dominates over substrate binding in the S2 to S4 sites (Sichler et al., 2002). Trypsin prefers Arg substrates over Lys substrates because Arg and Lys interact with the substrate binding pocket in different modes. The cyclic network of hydrogen bonds between the guanidinium group of P1 Arg and S1 Asp194 is the dominant feature of Arg substrate specificity. The chemical characteristics of the side-chain of Ser195 affects the specificity of trypsin towards P1 Lys through a critical hydrogen bond triad involving a water molecule, Ser195 Oδ and the substrate P1 Lys Nζ (Evnin et al., 1990). Substrate Lys is indirectly hydrogen bonded to the S1 site Asp194 via a water molecule.

The oxyanion hole. Gly198 in the oxyanion hole also plays a basic role in substrate binding by stabilizing the ground state and the transition state (Bobofchak et al., 2005). Gly198 is highly conserved in serine proteases, but in human trypsin-3 and brain trypsin-4 the residue at position 198 is Arg (Nyaruhucha et al., 1997). The conformation of the Arg198 side-chain prevents correct positioning of the amido hydrogen to form a hydrogen bond with the substrate. This feature together with His instead of Asp in position 218 is believed to provide the structural basis for the enhanced inhibitor resistance and binding affinity of substrates for human trypsin-3 and -4 (Katona et al., 2002).

The polypeptide binding site. The polypeptide binding site refers to the main chain of residues Ser215 –Asp218 which form an antiparallel beta sheet with the backbone of the P1 – P3 residues of peptide substrates. The beta sheet structure causes the side chains of the peptide substrate to point in opposite directions (Hedstrom, 2002, Sweet et al., 1974).

Loop structures. Outside the substrate binding pocket near the S1 binding site are two conserved loops called L1 and L2, respectively, which control the specificity of trypsin. These surface loops connect the walls of the S1 binding pocket and stabilize the transition state for hydrolysis by improving the orientation of bound substrates relative to the catalytic site (Hedstrom et al., 1994a). In addition, Tyr175 in a third surface loop has been identified as an additional specificity determinant (Hedstrom et al., 1994b, Perona et al., 1995). This kind of extended substrate binding accelerates catalysis.

It is thought that substrate discrimination occurs during the acylation step rather than during substrate binding. The structural basis for substrate discrimination in the acylation step is the ability of 1) P1-Arg or Lys to make favourable electrostatic interactions with Asp194 to enhance the accurate positioning of

Gly217 and of 2) loop L2 to uniquely specify the conformation of the conserved Gly217, which forms two main-chain hydrogen bonds with the P3 residue of the substrate promoting accurate scissile bond positioning in a discriminatory way (Hedstrom et al., 1992, Hedstrom et al., 1994a, Ma et al., 2005, Perona et al., 1995). In addition, there are five loops designated A to E. Loop C contacts the extended substrate on the N-terminal side of the scissile bond, whereas loops A, B, D and E interact on the leaving group side (Perona and Craik, 1997). Thus, S1 binding site contributes to substrate specificity for ester hydrolysis, whereas specific amide hydrolysis requires both the proper S1 binding site and more distal interactions such as with the loops next to the substrate binding pocket (Hedstrom et al., 1992).

The leaving group side interactions. The leaving group side interactions S' – P' are determined by surface loops (see above) (Bode and Huber, 1992, Perona and Craik, 1997). The role of S'1 to S'3 in substrate binding and catalysis in rat trypsin has been studied by active site mapping using nucleophile mixtures (Schellenberger et al., 1994). The most important contact in the S' subsites is a hydrogen bond between main-chain carbonyl oxygen S'2 – main-chain NH of the P'2 residue, where trypsin prefers positively charged residues. The P'1 and P'3 side-chains point in one direction and the P'2 side-chain in the opposite direction. Large amino acids residues in P'1 and P'3 can probably form contacts with the same region on the enzyme surface and most likely compete for contacts on the enzyme surface. In contrast, positive cooperativity is observed for specific P'2 and P'3 residues, as the P'2 and P'3 side-chains point tin opposite directions.

The P'2 side-chains bind to a region on the trypsin surface that is lined by His46 and Tyr154. Interestingly, in human pancreatic trypsin-1 and trypsin-2 Tyr154 is sulfated (Gaboriaud et al., 1996, Sahin-Tóth et al., 2006, Szilagyi et al., 2001) (IV). This feature, together with Asp at residue 218 (S4 site) is suggested to influence the selective binding of Kazal-type inhibitors to human trypsin-1 (Gaboriaud et al., 1996).

Catalysis

The physiological reaction catalyzed by trypsin is hydrolysis of peptide bonds on the carboxyl-terminal side of either arginine or lysine. Chemically the reaction is acyl transfer, in which trypsin stabilizes the tetraedral transition state typical to this reaction (Kraut, 1977). The mechanism is a base-catalyzed nucleophilic attack of the hydroxyl-O of Ser200 to the carbonyl-C of the substrate (Craik et al., 1987, Weiner et al., 1986). The so called catalytic triad or charge relay system – His63, Ser200 and Asp107 – is essential for the catalysis. It is part of an extensive hydrogen bonding network within the enzyme itself and with the substrate during catalysis. The strength of the hydrogen bonds changes during catalysis (Fodor et al., 2006).

Formation of a Michaelis complex. A Michaelis complex is formed upon substrate side-chain binding to the binding pocket: a hydrogen bond between the Oγ of Ser200 and Nε2 of His63 in the active site becomes sterically optimal for hydrogen transfer as the result of reorganization in the side-chain of Ser200 and movement of the imidazole ring of His63 (Ruhlmann et al., 1973). Then the Ser200 Oγ can form a covalent bond with substrate and donate a proton to His63. In addition, the side-chain of the substrate Lys residue becomes hydrogen-bonded to Asp194 via a water molecule. The longer side-chain of Arg in the substrate replaces the water molecule in the binding pocket and forms a direct hydrogen bond to Asp194 of trypsin (Bode et al., 1984, Craik et al., 1985, Weber et al., 1995).

The acylation step. After formation of the non-covalent Michaelis complex catalysis is then thought to proceed in two steps, which are simplified as follows. First, acylation of trypsin occurs by the nucleophilic attack of hydroxyl-O of Ser200 to the substrate P1 carbonyl-C resulting in a covalent bond. At

the same time, the hydroxyl-proton of Ser200 is transferred to the His63 Nε2 base. Hydrogen bonds are formed between the oxyanion hole and P1 carbonyl-C resulting in polarization of the carbonyl bond. All these bonds force the substrate to a tetrahedral oxyanion intermediate. Then, the imidazole ring of His63 can donate a proton to the leaving group amine on the substrate and the scissile bond is lengthened and broken. Trypsin acylation is most likely the rate-limiting step of the catalysis (Kossiakoff and Spencer, 1980, Kossiakoff and Spencer, 1981, Weiner et al., 1986). However, the deacylation step is only >3-fold faster than acylation (Bobofchak et al., 2005).

The deacylation step. After acylation a water molecule located in the catalytic site forms a hydrogen bond with His63. The water molecule acts as nucleophile by attacking the acyl-trypsin P1 carbonyl-C and donates a proton to His63. The formed new tetrahedral transition state breaks down as a proton is transferred from His63 to Ser200 and native trypsin is released (Weiner et al., 1986). The function of negatively charged Asp107, which is located on the opposite side of His63 in the active site, is believed by hydrogen bonding to force His63 in the proper tautomeric state, so that its proton is at Nδ1 and not at Nε2 (Ash et al., 1997, Frey et al., 1994, Kossiakoff and Spencer, 1981, Sprang et al., 1987). Amide bonds are very stable due to electron donation from the amide nitrogen to the carbonyl. It is estimated, that the rate of peptide bond hydrolysis is increased about 10^{10}-fold by serine protease catalysis as compared to the corresponding uncatalyzed reactions (Hedstrom, 2002).

Functions of trypsins

Digestion of food

Digestion of food is the main physiological function of pancreatic trypsin. Indeed, trypsin and chymotrypsin are considered the major workhorses of digestion. Trypsin-1 and -2 isoenzymes degrade dietary proteins in the duodenum either directly or indirectly by activation of other digestive enzymes such as chymotrypsinogen, procarboxypeptidase, phospholipase and proelastase (Neurath and Walsh, 1976, Travis and Roberts, 1969). Trypsin-3 does not activate zymogens, and thus degradation of dietary trypsin inhibitors appears to be the only role of pancreatic trypsin-3 (Sahin-Tóth, 2005, Szilagyi et al., 2001, Szmola et al., 2003) .

Intestinal alkaline sphingomyelinase digests dietary sphingomyelin generating multiple lipid messengers such as ceramide and sphingosine. Pancreatic trypsin has been shown to release intestinal alkaline sphingomyelinase from rat intestinal mucosa *in vivo*, thereby increasing the enzyme activity about 50 to 70% (Wu et al., 2004). The trypsin-induced dissociation was rapid and specific. By this means pancreatic trypsin would not only digest dietary proteins but indirectly also sphingomyelin.

Activation of protease-activated receptors

Protease-activated receptors (PAR-1, PAR-2, PAR-3 and PAR-4) are G protein-coupled receptors with seven transmembrane-spanning domains (Dery et al., 1998). PAR-2 is activated by trypsin-like enzymes like trypsin itself, acrosin and mast cell tryptase, whereas PAR-1, PAR-3 and PAR-4 are activated mainly by thrombin (Cottrell et al., 2003, Coughlin, 2005, Fox et al., 1997, Molino et al., 1997, Nystedt et al., 1994). To a lesser extent, human tissue kallikreins, cathepsin G, plasmin, granzyme A, and coagulation factors VIIa and Xa are able to activate PARs (Oikonomopoulou et al., 2006, Vergnolle et al., 2003). The PARs are irreversibly activated by proteolytic cleavage at the amino-terminal exodomain of the receptor. The new, unmasked amino terminus functions as a tethered ligand, docking intramolecularly with the body of the receptor to effect transmembrane signalling. Once ligated, PAR can activate intracellular G proteins and thus mediate extracellular signals to intracellular signalling pathways. Like other G protein-coupled receptors, PAR

signalling is rapidly attenuated by receptor desensitization, endocytosis, and/or receptor down-regulation (Grady et al., 1997, Ludeman et al., 2004). Trypsin has also been shown to affect PAR-2 ubiquitination, which is required for lysosomal trafficking of PAR-2 (Cottrell et al., 2003).

Protease-activated receptor-2. The gene encoding human PAR-2 has been cloned (Bohm et al., 1996) and PAR-2 has been found to be highly expressed in the pancreatic duct cells, kidney, intestine, liver, prostate, ovary, testes, heart, lung, skin, bladder, brain, and trachea, where it is found in epithelial and endothelial cells, and myocytes, fibroblasts, immune cells, neurons and glial cells (Bohm et al., 1996, D'Andrea et al., 1998, D'Andrea et al., 2000, D'Andrea et al., 2001, Macfarlane et al., 2001, Nguyen et al., 1999, Nystedt et al., 1994, Nystedt et al., 1995). Several functions of the PARs are involved in regulation of hemostasis, inflammation, pain, and tissue repair (Macfarlane et al., 2001). Functional PARs including PAR-2 have also been described in the central and peripheral nervous system suggesting regulative role for PARs and their activating proteases in various processes of the nervous system such as motor, secretory, vascular, nociceptive, inflammatory or regenerative processes (Vergnolle et al., 2003). PAR-2-mediated effects include increase in intracellular Ca^{2+}, effects of ion transport, cell proliferation, growth and adhesion, apoptosis, secretion, immunomodulation and mitogensis. PAR-signaling involves molecules like $G\alpha_i$, $G\alpha_q$, phospholipase Cβ (PLCβ), diacylglycerol (DAG), inositoltriphosphate (IP_3), NFκB, c-Fos, c-Jun, p38, and extracellular-signal regulated kinases (ERK1/2) (Steinhoff et al., 2005).

Pancreatic trypsins and PAR-2. Pancreatic trypsin-1 and -2 are potent activators of PAR-2, which is present at high densities on the luminal surfaces of pancreatic acinar cells, duct epithelial cells, and the intestine (Kong et al., 1997, Nguyen et al., 1999). PAR-2 activation stimulates cytokine production and regulates pancreatic exocrine function via a negative feedback loop (Hirota et al., 2006a, Maeda et al., 2005). Physiological concentrations of trypsin in the intestinal lumen (100 nmol/L) activates PAR-2 at the apical membrane of enterocytes and stimulates the generation of IP_3, arachidonic acid release and prostaglandin secretion (Kong et al., 1997).

In cultured dog pancreatic duct epithelial cells, trypsin can activate ion channels by cleaving and triggering PAR-2, which results in increased intracellular calcium concentration and subsequent stimulation of Ca^{2+}-activated Cl^- and K^+ channels (Nguyen et al., 1999). In the gastrointestinal track, PAR-2 mediated contractile responses have been reported, most likely via a mechanism involving Ca^{2+}-dependent K^+ channels (Cocks et al., 1999b). Furthermore, PAR-2 has been linked to the release of amylase from the acinar cells of the pancreas, and exocrine secretion from salivary, parotid and sublingual glands (Bohm et al., 1996, Kawabata et al., 2000, Kawabata et al., 2002, Nguyen et al., 1999). Thus, besides acting as digestive proteinase and activator of other digestive enzymes, pancreatic trypsin is also a signalling molecule regulating cells of the gastrointestinal track by activation of PAR-2.

Extra-pancreatic trypsins and PARs. Trypsin-4 has been suggested to activate PAR-2 and -4 and the inhibitor resistance of trypsin-4 has been postulated to promote prolonged PAR-mediated signaling in extra-pancreatic cells (Cottrell et al., 2004). Tumor-derived human epithelial cell lines from prostate (PC-3), colon (SW480 and Caco2), and airway (A549) have been found to express PAR-2, trypsinogen-4 and enteropeptidase. Expression of trypsinogen-4 and its activation by enteropeptidase induces a prompt increase in intracellular calcium in KNRK cells (a normal rat kidney [NRK] cell line transformed by Kirsten murine sarcoma virus) expressing human PAR-2, but not in nontransfected cells, suggesting that trypsin-4 is an activator of PAR-2 (Cottrell et al., 2003, Cottrell et al., 2004). However, studies with recombinant

trypsin isoforms revealed that the activity of trypsin-4 was completely unable to activate epithelial PAR-1 and-2. Instead, it weakly activated brain PAR-1 in human astrocytoma 1321N1 cells (Grishina et al., 2005).

Results from another group (Wang et al., 2006) revealed that trypsin-4 selectively induces transient Ca^{2+} mobilization in both rat astrocytes and retinal ganglion RGC-5 cells via activation of PAR-1. The activating cleavage site is Arg–Ser in PAR-1 and PAR-2, Lys–Thr in PAR-3, and Arg–Gly in PAR-4. Arg-Ser and Lys-Thr peptide bonds are readily cleaved by trypsin-3 and -4, so PAR-1, PAR-2, and PAR-3 are potential trypsin-4 substrates (Szepessy and Sahin-Tóth, 2006).

Trypsinogens in cancer

Proteolytic processing of ECM. As discussed above, trypsin degrades many ECM components (Koivunen et al., 1991a, Koshikawa et al., 1992, Moilanen et al., 2003, Stenman et al., 2005) but it is also a potent activator of several MMPs (Imai et al., 1995, Koivunen et al., 1989, Moilanen et al., 2003, Nyberg et al., 2002, Paju et al., 2001b, Sorsa et al., 1997, Umenishi et al., 1990). In addition to breakdown of ECM components and activation of proteinase cascades tumor-associated trypsins can modulate cancer cells by other mechanisms, too. Proteolytic processing of ECM exposes cryptic binding sites within ECM molecules, generates biologically active ECM fragments and affects the bioavailability and activity of sequestered growth factors and receptors (Liotta and Kohn, 2001).

Tumor-associated trypsin and PAR-2. Recent studies suggest a signalling function for tumor-associated trypsin as well as other proteinases. The binding of integrins to ECM proteins activates focal adhesion kinases (FAKs). These in turn interact with several intracellular signalling molecules. Stimulation of cellular growth, adhesion to fibronectin and vitronectin, and, when transplanted to nude mice, tumor production of human gastric carcinoma cells overexpressing trypsinogen-1 suggests that trypsin-1 contributes to disseminated growth of some cancer cells (Miyata et al., 1998). Integrin $\alpha_5\beta_1$-dependent cellular adhesion to fibronectin and proliferation of MKN-1 human gastric carcinoma cells was shown to be regulated by PAR-2 and G protein signalling induced by tumor-associated trypsin (Miyata et al., 2000).

Trypsin has been shown to be a potent growth factor for human colon cancer cells *in vitro* and the action is mediated by activation of PAR-2 and subsequent increase in intracellular Ca^{2+} concentration (Darmoul et al., 2001, Ducroc et al., 2002). The mechanism is dependent on MMP-mediated release of transforming growth factor-α (TGF-α), transactivation and phosphorylation of epidermal growth factor receptor (EGF-R) and subsequent activation of extracellular signal-regulated protein kinase 1/2 (ERK1/2) and cell proliferation (Darmoul et al., 2004). On the other hand, trypsinogen-4 has been hypothesized to possess a tumor-suppressive role in cancer progression as it has been shown to be silenced at the mRNA level by promoter methylation in several gastric adenocarcinomas and esophageal squamous cell carcinomas (Yamashita et al., 2003).

Trypsin-2 isolated from a colon carcinoma cell line has been shown to be more potent activator of PAR-2 than two different mast cell tryptases and almost equally effective as bovine pancreatic trypsin in an *in vitro* study (Alm et al., 2000). Interestingly, the PARs are up-regulated in cancer and inflammation (Borgono and Diamandis, 2004). Taken together, tumor-associated trypsins are potential *in vivo* activators of PAR-2.

Effects on the surrounding cells. Cells in the tumor microenvironment, i.e. mast cells, macrophages, endothelial cells, and vascular smooth muscle cells have been shown to express PAR-1 and PAR-2. These cells may act as proteolytic sensors to extracellular thrombin and trypsin, respectively, and thus enable a permissive environment for tumor growth and metastasis via an autocrine and/or paracrine cascade. PAR-1 and PAR-2 are also

detected on stromal fibroblasts surrounding metastatic tumor cells but not on fibroblasts surrounding benign, non-metastatic or normal epithelial cells (D'Andrea et al., 2001).

Angiogenesis. Tumor cell-activated endothelial cells produce trypsin, which has been suggested to contribute to tumor angiogenesis and tumor metastasis by activation of matrix metalloproteinases (MMPs) or direct matrix degradation (Koshikawa et al., 1997). On the other hand, many angiogenesis inhibitors are stored as cryptic fragments within larger precursor matrix molecules, and the regulation of proteolytic processing of extracellular matrix plays an important role in vascularization of tumors (Nyberg et al., 2005).

Other functions of trypsins

Paneth cells. The selective antibiotic activity of Paneth cell human α–defensin 5 and 6 (HD5 and HD6) is enhanced by tryptic processing. Unlike in the pancreas, only trypsin-2 and -3 are expressed at a 6:1 ratio in the Paneth cells of the small intestine, suggesting that Paneth cells express a distinct trypsin isoform pattern. Paneth cell-derived trypsin is suggested to be the processing proteinase of HD5 in vivo and trypsin activity seems to be carefully regulated by API and PSTI that also are present in the Paneth cells (Ghosh et al., 2002). Paneth cell trypsin could also be the activator of PAR-2 expressed on luminal surfaces of enterocytes of the human intestinal crypts.

Genital tract. Trypsin is widely distributed in the male genital tract and may play a physiological role in semen. Trypsin purified from human seminal fluid activates the proform of prostate specific antigen (PSA) (Paju et al., 2000), which cleaves semenogelins I and II in the sperm-entrapping gel forming after ejaculation (Lilja, 1985).

Central nervous system. Human trypsin-4 has recently been shown to selectively process two Arg-Thr peptide bonds in human myelin basic protein, which is the most abundant membrane protein in the central nervous system and an autoantigen in multiple sclerosis (Medveczky et al., 2006). Trypsin-4 has also been implicated in the increased production of glial fibrillary acidic protein (GFAP) and accumulation of β-amyloid in the brain of transgenic mice expressing trypsinogen-4 in neurons (Minn et al., 1998). However, the possible role of trypsin-4 in neurodegenerative diseases remains to be elucidated.

Ion channels. Acid-sensing ion channels (ASICs) are non-voltage-gated Na^+ channels of the epithelial Na^+ channel/degenerin family. They are almost ubiquitous in the mammalian nervous system and they are transiently activated by a rapid drop in extracellular pH (Krishtal, 2003). Several putative physiological roles of ASICs have been proposed, like pain receptor, modulation of synaptic transmission, memory and fear conditioning and mediation of cell injury in acidosis. Trypsin has been shown to cleave ASIC 1a in the N-terminal part of an extracellular loop *in vitro*, thereby shifting the pH-dependence of channel activation and inactivation to more acidic pH (Vukicevic et al., 2006). Trypsin has been demonstrated *in vitro* to cleave C termini of β– and γ–subunits of epithelial Na^+ channels (ENaC). This is believed to increase ENaC activity and be one of the physiological mechanisms of sodium channel regulation (Jovov et al., 2002).

Leucocyte adhesion. Trypsin has been reported to up-regulate the intercellular adhesion molecule-1 (ICAM-1), a key vascular endothelial adhesion molecule necessary for transport of leukocytes from the intravascular space into inflamed tissues (Hartwig et al., 2004). Up-regulation by trypsin occurs both in the rat pancreas and lung and it is associated with increases in leukocyte infiltration into the tissues and decreased perfusion of pancreatic microvasculature.

Airways. In preterm infants, the development of bronchopulmonary dysplasia (BPD) is associated with high pulmonary concentrations of trypsinogen-2 during the first two postnatal weeks (Cederqvist et al., 2003). In addition, infants with higher trypsinogen-2 to TATI

ratio subsequently developed BPD. The underlying mechanism remains unclear, but it has been suggested that trypsin degrades ECM directly, activates latent MMPs, or mediates inflammatory reactions via activation of PAR-2. Bronchial epithelial cells express both trypsin(ogen) and PAR-2 (Cocks et al., 1999a). Indeed, trypsin released from the epithelium can initiate brochorelaxation in the airways by activation of epithelial PAR-2 and is thus hypothesized to participate in prostanoid-dependent cytoprotection in the airways (Cocks et al., 1999a).

Other functions. Trypsin has been shown to participate in cardiovascular events via PAR-2 in animal models, but the physiological and pathophysiological role remains unclear (Macfarlane et al., 2001). In the skin, activation of PAR-2 by trypsin has been linked to pigmentation via action of prostaglandins and their receptors (Scott et al., 2004). In rat brain, trypsin has been shown to cleave the virus envelope fusion glycoprotein precursor hemagglutinin (HA_0) of human influenza A virus and the fusion glycoprotein precursor (F_0) of Sendai virus (Le et al., 2006). After virus infection in rat lungs the levels of TNF-α, trypsin-1 and MMP-9 mRNA, respectively, were significantly up-regulated (Yamada et al., 2006). These results suggest that trypsin in the brain might potentiate virus multiplication and progression of influenza-associated encephalopathy or encephalitis. Finally, a function as a "pipe-cleaner" has been proposed for trypsin produced by various types of epithelial cells, like those of the bile duct and the nephron of the kidney (Koshikawa et al., 1998).

Trypsin inhibitors

Several classes of inhibitors mimic the tetrahedral intermediate of the serine protease reaction and form stable tetrahedral adducts with the protease (Kraut, 1977) in a so called "canonical" substrate-like manner, where numerous polar and hydrophobic interactions between the protease and the inhibitor prevent rapid dissociation of the complex. Prolonged association of the enzyme and the inhibitor leads to an equilibrium between the cleaved and uncleaved forms of the inhibitor (Fodor et al., 2005, Fodor et al., 2006). However, not all inhibitors interact "canonically" (Rydel et al., 1990, Rydel et al., 1991), a covalent bond between the enzyme and the inhibitor is not necessary for inhibition, and the protease-inhibitor complex is not a fully tetrahedral adduct (Baillargeon et al., 1980, Richarz et al., 1980). The canonical inhibitors, like PSTI and API, are unrelated in structure but have in common a "primary binding segment", a flat-shaped loop that fits into the active-site cleft of cognate proteinase. All protein inhibitors of proteinases prevent access of (large) substrates to the catalytic site of the enzyme by steric hindrance. Endogenous protease inhibitors appear to be proteins, small non-protein inhibitors are produced by micro-organisms (Bode and Huber, 1992).

The first natural protease inhibitors were identified by Northrop and Kunitz as part of their protease studies in the 1930s with cattle pancreas (Kunitz and Northrop, 1935). An inhibitor was characterized as a polypeptide with a molecular weight of about 6000 Da, and that forms a reversible complex with trypsin in a molar ratio of 1:1. Today this inhibitor

Table 3. Trypsin inhibitors in human plasma

Inhibitor	Concentration (g/L)	MW (kDa)	Reference
α_2-macroglobulin	2 - 4	720	(Sottrup-Jensen, 1989)
α_1-proteinase inhibitor	1.3	51	(Carrell, 1986)
Inter-α-inhibitors	0.6 - 1.2	30 - 250	(Josic et al., 2006)
PSTI/TATI	5 - 20 x 10^{-6}	6.2	(Stenman et al., 1982)

is called basic pancreatic trypsin inhibitor (BPTI) or aprotinin, and it belongs to the Kunitz type inhibitor family. Human pancreas does not contain a Kunitz type inhibitor, but both human trypsin-1 and -2 are inhibited by BPTI in a 1:1 molar ratio (Figarella et al., 1975). Protease inhibitors are after albumin and immunoglobulins the third largest group of functional proteins comprising about 10% of total plasma proteins in vertebrates (Travis and Salvesen, 1983) (Table 3).

α_2-macroglobulin

α_2-macroglobulin (α_2M) and α_1-proteinase inhibitor (API, also called α_1-antitrypsin) are the major protease inhibitors in human plasma (Laskowski and Kato, 1980) (Table 3). Human α_2M is a large (MW 720 000 Da) glycoprotein, composed of four identical subunits (Sottrup-Jensen, 1989). It is a non-specific protease inhibitor, which controls the activity of proteinases not only by active site-directed inhibition but also by steric shielding and rapid clearance. Specific limited proteolysis of α_2M at a site called the bait region results in a conformational change in α_2M leaving the protease irreversibely bound to α_2M (Barrett et al., 1979, Borth, 1992, Bretaudiere et al., 1988). One α_2M molecule can trap one or two proteinase molecules (Sottrup-Jensen, 1989). Protease-α_2M complexes are rapidly eliminated from the circulation by LDL-receptor-related protein mediated endocytosis (Sottrup-Jensen, 1989) primarily by hepatocytes (Feldman et al., 1985).

α_1-proteinase inhibitor

Apart from α_2M, the most abundant human plasma proteinase inhibitors are serpins (serine proteinase inhibitors). The serpins share a conserved structure and employ a unique irreversible suicide substrate-like inhibitory mechanism. Thirty four human serpins, including α_1-proteinase inhibitor (API) have already been identified (Gettins, 2002). API is the serpin present at the highest concentration in human plasma and it is mainly produced by the liver (Carrell, 1986). API is able to inhibit several serine proteases, but the regulation of neutrophil elastase is considered to be its main physiological function (Beatty et al., 1980, Travis and Salvesen, 1983).

Human API (MW 51 000 Da) forms complex with trypsin in a 1:1 molar ratio. The proteinase first forms a noncovalent Michaelis complex with API. Subsequent peptide bond hydrolysis of the reactive center loop results in formation of acyl-enzyme intermediate and insertion of the reactive center loop into a β–sheet. Upon complete loop insertion the proteinase is translocated and compressed against the base of API, its active site is grossly distorted and hence inactivated (Huntington et al., 2000, Silverman et al., 2001).

Proteinases complexed to API can be degraded by other proteinases (Kaslik et al., 1995, Stavridi et al., 1996). This may be a faster way of proteinase elimination from the circulation than the SEC (serpin-enzyme complex) receptor-based uptake and intracellular degradation of proteinase-API complexes (Perlmutter et al., 1990, Pizzo, 1989, Pratt et al., 1988). API inhibits trypsin-2 ten times faster than trypsin-1, and it has been suggested to control trypsin-1 activity *in vivo* when α_2M is already saturated (Vercaigne-Marko et al., 1989). In this case API would have a significant role in the inhibition of trypsin-2 under physiological conditions and of trypsin-1 under pathological conditions.

Inter-α-inhibitors

Inter-α-inhibitor proteins comprise a family of serine proteinase inhibitors found at relatively high concentrations in human plasma, i.e. 0.6 to 1.2 g/L (Josic et al., 2006). Inter-α-trypsin inhibitor (ITI) was first characterized and isolated from human plasma in the 1960s (Heimburger et al., 1964, Steinbuch and Loeb, 1961). It was initially characterized as a zinc-containing glycoprotein that inhibits trypsin and chymotrypsin by forming 1:1 complexes (Aubry and Bieth, 1976). ITI was shown to be structurally related to the Kunitz family of inhibitors and homologous to bovine

pancreatic trypsin inhibitor BPTI (Wachter and Hochstrasser, 1981), but its antiproteinase function is relatively weak.

The inter-α-inhibitor proteins consist of heavy chains, H1, H2, H3 and/or H4 (65, 70, 90 and 120 kDa, respectively) and/or a 30 kDa light chain called bikunin. The genes encoding the subunits have been characterized (Nishimura et al., 1995, Salier, 1990). Uncomplexed bikunin, which has two Kunitz-type inhibitory domains, inhibits several serine proteinases including trypsin, plasmin, elastase and cathepsin B (Josic et al., 2006, Salier et al., 1996). The 250 kDa inter-α-inhibitor, previously called ITI, contains three subunits, two heavy chains H1 and H2 and bikunin. The 125 kDa pre-α-inhibitor (PαI), previously known as pre-α-trypsin inhibitor, contains two subunits, heavy chain H3 and bikunin. (Josic et al., 2006). The chains are covalently bound via a protein – glycosaminoglycan – protein bridge, where chondroitin 4-sulfate is the glycosaminoglycan. Bikunin contains an N-linked oligosaccharide and a chondroitin sulfate chain (Josic et al., 2006, Salier et al., 1996).

Studies in mice suggest that ITI acts as a shuttle by transferring proteinases to other plasma proteinase inhibitors like α_2M and API for clearance, and that ITI modulates the distribution of proteinase among inhibitors (Pratt and Pizzo, 1986, Pratt et al., 1987). On the other hand, a so-called von Willebrand type-A, multicopper oxidase and bradykinin-like domains have been identified in the heavy chains, suggesting several other functions, like a role in inflammation and maintenance of extracellular matrix stability and integrity through hyalyronic acid-binding (Bost et al., 1998, Salier et al., 1996).

PSTI or TATI

SPINK1 gene. PSTI is Kazal-type trypsin inhibitor originally purified from bovine pancreas from a side-fraction in a commercial insulin process (Kazal et al., 1948). The sequence of human PSTI was identified in 1977 (Bartelt et al., 1977) and today the single human PSTI gene (serine protease inhibitor Kazal type 1 or *SPINK1* gene) has been characterized. It is 7.5 kb long, separated into four exons and is located on chromosome 5 (Horii et al., 1987).

The genomic PSTI gene has neither the mammalian pancreas-specific common cis-acting regulatory sequence (Walker et al., 1983) nor the typical promoter sequences TATA, CAAT nor GC boxes, but the sequences ATAT and CAATCAAT are positioned in the promoter region of the gene (Horii et al., 1987). It has been suggested that the sequence CAATCAATAAC that is present in two novel 5' cis-acting elements in the promoter region of the gene functions as a pancreas-specific element (Yasuda et al., 1998). A 40-bp IL-6-responsive element, that is conserved among various acute phase genes, has been identified in the PSTI gene in hepatoma cells (Yasuda et al., 1993).

Biochemical properties of PSTI/TATI. The *SPINK1* gene product consists of 79 AAs including a 23 AA signal peptide. Mature PSTI is a 56 AA polypeptide with a molecular weight of 6242 Da containing three intra-chain disulphide bridges. PSTI, or tumor-associated trypsin inhibitor (TATI), isolated from urine of a patient with ovarian cancer (see below) was found to be microheterogenous in charge the pI of the main component being 5.8 (Huhtala et al., 1982). Four forms of PSTI have been purified in human pancreatic juice (Kikuchi et al., 1985). PSTI/TATI is cleared from circulation by excretion into urine with a half-life of six minutes (Marks and Ohlsson, 1983). In fact, serum PSTI/TATI can also be used as a marker for renal function (Tramonti et al., 2003).

PSTI is synthetized and secreted together with trypsinogen by pancreatic acinar cells. The molar ratio of trypsinogen to PSTI in human pancreatic juice is about 5:1 (Hirota et al., 2006a, Rinderknecht, 1986, Rinderknecht, 1993) representing an amount equivalent to 0.1 to 0.8% of the total protein in pancreatic

juice (Pubols et al., 1974). The reactive site of human PSTI is residue Lys41, that serves as a specific target substrate for trypsin (Bartelt et al., 1977).

Serum levels of PSTI/TATI. TATI was first isolated from urine of an ovarian cancer patient (Stenman et al., 1982) and was later shown to be identical to PSTI (Huhtala et al., 1982). The concentration of TATI in normal serum is 5 to 20 µg/L and that in urine 5 to 50 µg/L as measured by radioimmunoassay. Elevated levels have been observed in urine from patients with ovarian, cervical and endometrial cancer, as well as in the amniotic fluid from 14 to 16 weeks of pregnancy (Stenman et al., 1982). As measured by another radioimmunoassay, serum PSTI level in healthy individuals ranged from 5.4 to 16.0 µg/L (Kitahara et al., 1980). Normal serum levels of TATI were found in the serum and urine of pancreatectomized patients (Halila et al., 1985) suggesting that pancreatic acinar cells are not the main source of PSTI/TATI in humans.

Inhibition. PSTI is a strong, reversible trypsin inhibitor, which inhibits both trypsin-1 and -2 in an equimolar ratio. It is gradually degraded and released from trypsin (Figarella et al., 1975, Laskowski and Wu, 1953) by cleavage of the peptide bonds Lys41-Ile42, Arg67-Gln68, Arg28-Glu29, Arg65-Lys66 and Lys75-Ser76 (Kikuchi et al., 1989, Schneider and Laskowski, 1974, Schneider et al., 1973). Both human and dog PSTI-trypsin complexes dissociated rapidly when added into serum *in vitro* (Eddeland and Ohlsson, 1978). The released trypsin was mainly bound by serum α_2M and to a lesser extent to API.

Intravenous injection of PSTI-trypsin complexes into dogs resulted in a similar rapid dissociation of the complexes. The major part of the injected radioactive trypsin was bound by α_2M and API. The released PSTI disappeared rapidly from the circulation into urine and into the whole extracellular fluid volume (Eddeland and Ohlsson, 1978). The peptide bonds Lys41-Ile42, Arg67- Gln68, Arg28-Glu29, and Lys75-Ser76 have also been shown to be cleaved by trypsin-3 (Szmola et al., 2003).

The function of PSTI in the mucus-producing cells in the gastrointestinal tract is suggested to protect the mucus from digestion by luminal proteinases within the stomach and colon and to stimulate epithelial repair (Freeman et al., 1990, Marchbank et al., 1998). PSTI/TATI is also an efficient inhibitor of acrosin (Huhtala, 1984) suggesting a role in reproduction.

In PSTI deficient *(Spink3-/-)* mice, autophagic degeneration of acinar cells started from day 16.5 after coitus, resulting in rapid onset of cell death in the pancreas and duodenum, and finally death of the test animals 14.5 days after birth (Ohmuraya et al., 2005). The same researchers reported later (Ohmuraya et al., 2006) that trypsin activity could be detected in pancreatic acinar cells of *Spink3-/-* mice at 0.5 and 1.5 days after birth. On the contrary, trypsin activity was not detected in pancreatic acinar cells of *Spink3+/+* and *Spink3+/-* mice. Thus, the loss of PSTI resulted in failure to control trypsin activation in acinar cells in mice leading to excessive autophagy in the acinar cells.

Extrapancreatic expression. The physiological role of PSTI was initially thought to solely prevent premature activation of pancreatic proteases, especially trypsinogen (Pubols et al., 1974, Rinderknecht, 1986). However, TATI as well as trypsinogen (see above) are also expressed in several other normal tissues like the gastrointestinal tract (Bohe et al., 1986, Bohe et al., 1988, Bohe et al., 1992, Bohe et al., 1997, Freeman et al., 1990, Shibata et al., 1986), gall bladder and biliary tract, breast, kidney and urinary tract, spleen, epithelial cells of the skin, liver, lung, the brain and vascular endothelial cells (Fukayama et al., 1986, Lukkonen et al., 1999, Marchbank et al., 1996) suggesting an important role for both TATI and trypsinogen in tissues other than the pancreas.

PSTI/TATI in cancer. The increase of serum PSTI/TATI found in connection with malignant diseases is probably caused by production in the cancer cells, but the acute-phase reaction can also contribute. PSTI/TATI has been shown to be expressed in several cancers, including pancreatic, colorectal, gastric, lung, ovarian, renal cell, and bladder cancers (Diggle et al., 2003, Haglund et al., 1986, Higashiyama et al., 1990a, Higashiyama et al., 1990b, Huhtala et al., 1982, Huhtala et al., 1983, Jarvisalo et al., 1993, Lukkonen et al., 1999, Ohmachi et al., 1993, Paju et al., 2004, Paju et al., 2007, Pasanen et al., 1995, Piantino and Arosaio, 1991, Tomita et al., 1987).

TATI has been shown to be prognostic factor in ovarian cancer (Venesmaa et al., 1994), bladder cancer (Kelloniemi et al., 2003), hepatocellular carcinoma (Lee et al., 2007), and renal cell carcinoma (Paju et al., 2001a). The function of TATI in cancer is thought to be the same as in the pancreas, i.e. the inhibition of trypsin produced by the tumor cells (Stenman et al., 1991). The finding that trypsinogen is expressed in both malignant and benign bladder epithelium, whereas TATI expression decreases with increasing stage and grade, suggests balanced expression of trypsinogen and TATI in normal tissue, but disruption of this balance in tumor progression (Hotakainen et al., 2006). Interestingly, high TATI expression in gastric cancer tissue seems to correlate with a favourable prognosis for the patient (Wiksten et al., 2005), but in prostate cancer high TATI expression is associated with aggressive disease (Paju et al., 2007).

Acute phase reaction. PSTI has been suggested to be an acute-phase protein and to be induced by inflammatory cytokines (Yasuda et al., 1990). The PSTI levels in serum increase in connection with severe inflammation, tissue destruction and major surgery (Lasson et al., 1986, Matsuda et al., 1985, Ogawa et al., 1985, Ogawa et al., 1988). PSTI-production in pancreatic acinar cells is not regulated by the acute-phase process, as suggested by analyzing PSTI, trypsinogen-1 and α_1-antichymotrypsin, another acute-phase reactant, in plasma and pancreatic juice after partial pancreatic resection (Jonsson et al., 1996).

In response to inflammatory cytokines, the liver produces several acute-phase proteins that are proteinase inhibitors. There is some evidence indicating that the liver might also be a source of PSTI in acute-phase reactions in humans. In cultured human hepatoblastoma cells, PSTI production is stimulated by IL-6 (Yasuda et al., 1990) and an IL-6-responsive element has been identified in the PSTI gene (Yasuda et al., 1993). Furthermore, PSTI is produced by hepatocellular cancer cells (Ohmachi et al., 1993) and the secretion of PSTI by human hepatocellular cancer cell line is substantially increased in the presence of cytokine-producing mononuclear white blood cells (Jonsson et al., 1996). Acute-phase proteins are thought to prevent non-specific tissue damage caused by proteinases released from activated immune and phagocytic cells (Roberts et al., 1995).

Polyamines

Polyamines, like spermidine and spermine, are needed for normal cellular growth and differentiation (Nitta et al., 2002). Exocrine pancreas has the highest spermidine concentration in the mammalian body, and it is thought to be related to the high rate of protein synthesis in this tissue. Activated polyamine catabolism in transgenic rats results in severe acute pancreatitis (Alhonen et al., 2000) and is associated with intracellular trypsinogen activation (Hyvonen et al., 2006). In the pancreas, polyamines have been localized in zymogen granules. Thus, it is possible that polyamines directly inhibit proteinase activity, and that their depletion thus would result in a direct activation of proteolytic enzymes (Hyvonen et al., 2006).

Pancreatitis

In the normal pancreas, the hazardous effects of proteinase activity are controlled by regulated expression and secretion, storage of zymogens within membrane-bound granules,

regulated activation of the proenzymes, specific degradation and autolysis of the active proteinases, inhibition of their proteolytic activity, and controlled lysosomal degradation and autodegradation of digestive enzymes of damaged cells (Logsdon, 2001). However, pancreatitis is a necrotic and inflammatory process of the pancreas, where, with the exception of infectious pancreatitis, premature activation of trypsinogen and other digesitive pancreatic zymogens within or near the pancreas start digesting the pancreas itself (Kloppel and Maillet, 1993, Kloppel, 2007). Pathophysiologically, autodigestion and inflammation may be caused by either increased proteolytic activity or decreased proteinase inhibition.

Pancreatitis can be acquired or hereditary, acute or chronic (Kloppel and Maillet, 1993). Ethanol abuse and gallstones account for about 80% of acute pancreatitis cases (Le Moine et al., 1994, Lee et al., 1992). Chronic pancreatitis is usually caused by many years of alcohol abuse, ductal obstruction, exposure to cigarette smoke or volatile hydrocarbons, or can be autoimmune or hereditary (Chari, 2007, McNamee et al., 1994, Talamini et al., 1996).

Especially in acute pancreatitis (I) (Borgström and Andren-Sandberg, 1995, Kimland et al., 1989, Petersson et al., 1999), but also in pancreatic cancer, chronic alcoholism and chronic pancreatitis (Borgström and Andren-Sandberg, 1995, Rinderknecht et al., 1979, Rinderknecht et al., 1985) the proportion of serum trypsinogen-1 and -2 immunoreactivity becomes reversed, suggesting nonparallel secretion of the trypsinogen isoforms in pancreatic disease. By using recombinantly produced trypsinogen-1 and -2, Kukor et al. (Kukor et al., 2003) demonstrated that the up-regulation of trypsinogen-2 in potential pathological conditions significantly limits trypsin generation. In conditions modeling those of pancreatic juice (1 mmol/L Ca^{2+}, pH 8), trypsin generation by autoactivation or enteropeptidase activation was not affected significantly by the ratio of the two isoforms due to faster autodegradation of trypsinogen-2 and trypsin-2. However, trypsin generation was markedly diminished under conditions that modeled cytoplasm or acidic vesicles (50 μmol/L Ca^{2+}, pH 5) by an increased ratio of trypsinogen-2, because acidic pH inhibited activation of trypsinogen-2, whereas it stimulated autoactivation of trypsinogen-1. This suggests that, as a defensive mechanism, acinar cells increase secretion of trypsinogen-2 in pancreatic diseases, thereby decreasing the chance for premature trypsinogen activation inside the pancreas, while maintaining acceptable trypsin function in the duodenum (Kukor et al., 2003).

Hereditary pancreatitis

Hereditary pancreatitis (HP) is caused by mutation(s) inducing premature intracellular activation of proteolytic enzymes, especially trypsin. The phenotypic features of hereditary pancreatitis include autosomal dominant inheritance, high penetrance (80%), intermittent attacks of acute pancreatitis usually beginning in childhood, and frequent progression of the disease to chronic pancreatitis (Gorry et al., 1997). Patients with hereditary pancreatitis, especially those with a paternal inheritance pattern, have a high risk of developing pancreatic cancer several decades after the onset of pancreatitis (Lowenfels et al., 1997).

A relationship between the onset of pancreatitis and a mutation in the trypsinogen-1 gene was initially reported in 1996 (Whitcomb et al., 1996). Since then, several mutations in the trypsinogen-1 *(PRSS1)*, PSTI *(SPINK1)*, and cystic fibrosis transmembrane conductance regulator *(CFTR)* genes have been found to be associated with chronic pancreatitis (Keiles and Kammesheidt, 2006). An up-to-date database of published *PRSS1*, *PRSS2* and *SPINK1* variants can be found at www.uni-leipzig.de/pancreasmutation.

Mutations in the PRSS1 gene. The AA substitutions in trypsinogen-1 are located in the activation peptide, the N-terminal part of

trypsin, and in the longest peptide segment not stabilized by disulfide bonds between Cys64 and Cys139, which also encompasses the calcium-binding loop. The mutations appear to be associated with enhanced activation (Chen et al., 2003a, Feréc et al., 1999, Gorry et al., 1997, Pfutzer et al., 2002, Sahin-Tóth and Tóth, 2000, Sahin-Tóth, 2000, Sahin-Tóth, 2001, Simon et al., 2002, Teich et al., 2000, Teich et al., 2004, Whitcomb, 1999), inhibition of autolysis, or enhanced stabilization (Le Maréchal et al., 2001, Pfutzer et al., 2002, Sahin-Tóth, 2001, Simon et al., 2002, Whitcomb et al., 1996).

The most frequent mutation in HP worldwide is Arg122His, which eliminates the autolysis site of trypsin-1 and alters autoactivation and autodegradation of trypsinogen-1 (Simon et al., 2002). Unlike all other known trypsinogens, human trypsinogen-1 contains Asn at position 29. With the exception of human trypsinogen-2 that has Ile at position 29, all other mammalian trypsinogens contain Thr29 (Rypniewski et al., 1994). The second most frequent HP-associated mutation is Asn29Ile in human trypsinogen-1 (Gorry et al., 1997). Other mutations of Asn29 have also been shown to affect autoactivation (Sahin-Tóth, 2000). Chymotrypsin C –mediated processing of the trypsinogen-1 activation peptide is increased 4-fold by the mutation Ala16Val, resulting in accelerated trypsinogen activation *in vitro* (Nemoda and Sahin-Tóth, 2006).

A novel mechanism underlying HP was suggested by Teich et al. (Teich et al., 2004). The activation of trypsinogen-2 by mutated Glu79Lys-trypsin-1 was increased two-fold, and HP could thus be caused by increased transactivation of trypsinogen-2 by mutated trypsin-1. Furthermore, triplication of a ~605 kb gene segment containing the *PRSS1* gene on chromosome 7 seems to result in increased trypsin expression through a gene dosage effect causing HP (Le Maréchal et al., 2006).

Mutations that protect against pancreatitis are very rare but have been reported. Mutations Tyr37X and IVS2+1G>A that result in non-functional product of the *PRSS1* gene were found in two of 55 alcoholics without chronic pancreatitis, respectively (Chen et al., 2003b). Furthermore, mutation Gly191Arg results in degradation-sensitive trypsinogen-2 (Witt et al., 2006).

It has been suggested that gene conversion is a likely cause of PRSS1 missense mutations associated with HP (Chen and Feréc, 2000a, Chen and Feréc, 2000b). Gene conversion is a process where a functional gene is converted into a mutant one by unidirectional transfer of genetic information from a homologous, non-functional donor gene to the functional acceptor gene (Baltimore, 1981, Chen et al., 2007). The genes T4 to T8 are organized in tandem repeats and share 91% overall sequence homology (Rowen et al., 1996), which render them prone to gene conversion events. The presence of several donor sequences in genes T6, T7, T8 and T9 for Arg122His, Asn29Ile and Ala16Val mutations, respectively, is strongly suggestive of these mutations being caused by gene conversion events. Furthermore, chi-like and palindromic sequences are frequently observed in the vicinity of potentially converted gene fragments (Collier et al., 1993, Giordano et al., 1997, Patrinos et al., 1998). Such sequences are also found in the 3' and/or 5' boundaries of the Arg122His, Asn29Ile and Ala16Val mutations (Chen and Feréc, 2000a, Chen and Feréc, 2000b).

Mutations in the SPINK1 gene. Mutations and polymorphisms in the *SPINK1* gene are also associated with HP. These include single-nucleotide substitutions, microinsertions/deletions (Chen et al., 2000, Kiraly et al., 2007, Le Maréchal et al., 2004, Pfutzer et al., 2000, Witt et al., 2000) and a large 1336 bp deletion involving the promoter region and exon 1 of *SPINK1* (Masson et al., 2006).

The Asn34Ser mutation (Witt et al., 2000) is the most common HP-associated variant in the PSTI gene. Asn34 is located close to Lys18, the target P1 residue of trypsin, and the mutation has been suggested to lead to decreased inhibitory capacity of PSTI by affecting the

conformation of the active site (Witt et al., 2000). On the other hand, biochemical and surface-plasmon-resonance (SPR) analysis of recombinant Asn34Ser mutant and wild type PSTI showed no difference in binding (Hirota et al., 2003). Thus, the Asn34Ser mutation has been suggested to act more like a disease susceptibility factor, possibly by lowering the threshold for pancreatitis caused by other genetic or environmental factors (Masson et al., 2006, Pfutzer et al., 2000, Schneider et al., 2002). Interestingly, the Asn34Ser mutation always co-segregates with intronic mutations and altered splicing has been suggested to underlie the predisposition to HP (Kuwata et al., 2002).

The Met1Thr mutation eliminates the start codon of PSTI leading to an overall loss of PSTI expression (Witt et al., 2000). The Arg67Cys mutation has been suggested to cause massive conformational alterations in the protein, probably by a novel intra- or intermolecular disulfide bond, as the mutant recombinant PSTI isoforms lost their reactivity with an anti-PSTI (wild type) antibody (Hirota et al., 2003). Signal peptide variants that have been demonstrated to impair secretion of PSTI and destine the inhibitor for rapid intracellular degradation are also associated with HP (Kiraly et al., 2007).

Cystic fibrosis transmembrane conductance regulator gene mutations. Mutations in the cystic fibrosis transmembrane conductance regulator gene *(CFTR)*, located on chromosome 7q31 (Riordan et al., 1989), lead to exocrine glandular dysfunction and cystic fibrosis, but are also associated with chronic pancreatitis (Audrezet et al., 2002, Sharer et al., 1998). The first organ to be affected in phenotypical severe CF is the exocrine pancreas (Schwiebert et al., 1998). In a cohort of 134 patients with chronic pancreatitis Sharer et al. (Sharer et al., 1998) found – and the results have been confirmed by others (Cohn et al., 1998, Ockenga et al., 2000) – that the frequency of *CFTR* mutation was higher than expected. Thus, mutations of the *CFTR* gene are a risk factor for chronic pancreatitis.

The *CFTR* protein is present at high levels in intralobular and proximal ductular epithelia of the pancreas and at low levels in acinar cells. There it maintains the solubility of the secreted enzymes by mediation of the secretion of bicarbonate-rich alkaline fluid (Shumaker et al., 1999). Impaired solubility of pancreatic juice may thus be responsible for the increased risk of pancreatitis. Decreased activity of the mutated CFTR protein has also been suggested to interact with mutations in PSTI or trypsinogen-1 gene (Hirota et al., 2006b).

HP and acinar cell viability. The role of trypsinogen-1 in pancreatitis has been studied by the expression of wild type active trypsin-1, trypsinogen-1 and trypsinogen-1 bearing HP-associated mutations in the pancreatic acinar cell line AR4-2J (Gaiser et al., 2005). AR4-2J cell viability was reduced dose-dependently by transfection with a vector coding for functional trypsin-1, trypsinogen mutants Ala16Val, Asp22Gly, Lys23Arg and Arg122His. Wild type trypsinogen-1 had no effect on cell viability. Caspase-3 activity was shown to be significantly higher in cells expressing active trypsin-1 or the Arg122His trypsinogen-1 than in cells expressing wild type trypsinogen-1. Furthermore, caspase-3 activity was reduced in the presence of trypsin inhibitor. These findings suggest that expression of HP-associated mutations result in a stress strong enough to induce apoptosis in AR4-2J cells probably due to intracellular occurence of active trypsin. Thus, the sensibility of acinar cells to intracellular trypsin activity leading to apoptosis might have a protective effect in the pancreas (Gaiser et al., 2005).

Clinical value of trypsinogen determinations

In serum of healthy subjects, the average concentration of trypsinogen-1 has been reported to be 15 to 26 µg/L (I) (Borgström and Ohlsson, 1976, Florholmen et al., 1984b, Geokas et al., 1979) and that of trypsinogen-2 5.5 to 17 µg/L (I) (Largman et al., 1978).

Cystic fibrosis. The exocrine pancreas is affected in cystic fibrosis (CF), a disease caused by mutations in a chloride channel encoded by cystic fibrosis transmembrane conductance regulator gene *(CFTR)* (Aleksandrov et al., 2007, Riordan et al., 1989). Pancreatic damage begins *in utero*, which can be identified in neonates on the basis of elevated blood concentrations of pancreatic enzymes, especially trypsinogen (Sharer et al., 1998 and references therein). Transiently elevated serum levels of immunoreactive trypsinogen (IRT) have been found to be associated with the presentation of typical as well as atypical CF (Castellani et al., 1997). Determination of serum IRT in neonates is used as a test for screening for CF in many countries (Rock et al., 2005). Another test for CF is the sweat chloride test. A positive result with IRT and/or sweat chloride test is confirmed by gene mutation analysis (Spence et al., 1993).

Acute pancreatitis. Trypsinogen, trypsinogen activation peptide (TAP) and trypsin-API complexes in body fluids have been found to be increased in acute pancreatitis and can thus be used as diagnostic markers (I) (Borgström and Ohlsson, 1978, Borgström et al., 2002, Brodrick et al., 1979, Dubick et al., 1987, Elias et al., 1977, Florholmen et al., 1984b, Geokas et al., 1979, Gudgeon et al., 1990, Hedström et al., 1994, Hedström et al., 1996c, Hedström et al., 1996d, Petersson and Borgström, 2006, Petersson et al., 1999, Sainio et al., 1996, Tenner et al., 1997). Commercial radioimmunoassays, enzyme immunoassays and immunofluorometric assays for trypsinogen as well as TAP ELISA assay are available. A rapid dipstick screening test for pancreatitis, based on immunochromatographic measurement of urinary trypsinogen-2, has been developed (Hedström et al., 1996b, Kemppainen et al., 1997) and shown to detect acute pancreatitis more accurately than routinely used quantitative serum or urinary amylase determinations. However, serum amylase, often supplemented with serum lipase has remained the cornerstone laboratory test for diagnosis of acute pancreatitis in hospitals.

Allograft rejection and malnutrition. Serum trypsinogen-2 is shown to be accurate and sensitive diagnostic marker for rejection and inflammation occurring in the pancreatic allograft following pancreas-kidney transplantation (Douzdjian et al., 1994, Lieberman et al., 1997, Perkal et al., 1992). Co-monitoring serum amylase and trypsinogen-2 increased the specificity and diagnostic accuracy of the biochemical tests (Lieberman et al., 1997). Furthermore, increased levels of serum trypsinogen-1 have been reported in acutely malnourished infants and children (Durie et al., 1985). Improvement in nutritional status reverted trypsinogen-1 levels to normal.

Malignancies. Trypsinogens are associated with several malignancies and they could be used as diagnostic and prognostic factors for some cancers. However, the use of these tests is very limited. TAT-2 complexed with API has been shown to be a strong prognostic marker in advanced epithelial ovarian cancer (Paju et al., 2004). Immunohistochemically detected trypsin has been shown to correlate with disease recurrence and poor prognosis in human colorectal cancer (Yamamoto et al., 2003) and esophageal squamous cell carcinoma (Yamamoto et al., 2001). In non-small cell lung cancer (NSCLC) up-regulation of trypsinogen IVb *(PRSS3)* and trypsinogen C (TRY6) gene expression are predictors of distant metastasis and survival as revealed by microarray analysis (Diederichs et al., 2004).

Serum trypsinogen-2 has high accuracy in differentiating between cholangiocarcinoma and primary sclerosing cholangitis (Lempinen et al., 2007). Thus, it is a useful marker diagnosing patients with cholangiocarcinoma, and it is superior to serum tumor markers CA 19-9 and CEA.

Post-translational modification of proteins

The explosion of genetic information has increased our knowledge of living systems enormously. However, the structure of a mature protein is not dependent solely upon its gene, but also on post-translational modifications (PTMs). Chemical, biochemical, and enzymatic PTM of proteins to specific amino acid residues are common as over 200 variant amino acid residues have been detected (Creighton, 1984). They include disulfide bridge formation, glycosylation, proteolysis, phosphorylation, acylation, adenylation, farnesylation, ubiquitination, sulfation, amidation, oxidation, methylation, nitration, citrullination, isoprenylation, and palmitoylation, among others. These modifications affect the properties of proteins in many ways, i.e. activity, lifespan and protein-protein interactions (Hochrainer and Lipp, 2007, Li and Shang, 2007, Omary et al., 2006, Vader et al., 2006, Vervoorts et al., 2006, van der Horst and Burgering, 2007).

Tyrosine *O*-sulfation

Tyrosylprotein sulfotransferase. Sulfate trioxide (SO_3) may be covalently bound to the hydroxyl group on the side-chain of tyrosine and each sulfate moiety increases the molecular mass of the protein by 79.957 Da (Kehoe and Bertozzi, 2000). Protein tyrosine *O*-sulfation was first observed by Bettelheim in bovine fibrinopeptide B in 1954 (Bettelheim, 1954). Later, it was shown to be a ubiquitous protein modification (Huttner, 1982) mediated by tyrosylprotein sulfotransferase (TPST, EC 2.8.2.20) (Huttner, 1987, Lee and Huttner, 1983). TPST catalyzes the transfer of sulfate from the universal sulfate donor 3'-phosphadenosine 5'phosphosulfate (PAPS) to the hydroxyl group of tyrosine residues of proteins to form a tyrosine O^4-sulfate ester and 3',5'-ADP (Lee and Huttner, 1983). TPST is an integral membrane glycoprotein present in two forms (TPST-1 and TPST-2) in the *trans* Golgi network, and the two forms are coexpressed in many species, tissues and cell lines throughout the plant and animal kingdom examined so far (Baeuerle and Huttner, 1987, Beisswanger et al., 1998, Huttner, 1987, Lee and Huttner, 1985, Moore, 2003, Niehrs and Huttner, 1990, Ouyang and Moore, 1998, Ouyang et al., 1998, Ouyang et al., 2002, Vargas et al., 1985, William et al., 1997, William et al., 1997).

Predicted tyrosine sulfation sites. The Golgi localization and the luminal active site orientation of TPST-1 and -2 predict that tyrosine *O*-sulfation occurs only on proteins that transit through the *trans* Golgi network and there is no evidence of violation of this rule (Moore, 2003). Not only secreted proteins but also membrane-bound proteins are equally likely to be sulfated (Hille and Huttner, 1990, Hille et al., 1990). There is no sequon for tyrosine *O*-sulfation *per se*, but consensus features predicting tyrosine sulfation have been proposed.

First, the presence of acidic amino acids like aspartic or glutamic acid at position -1 and at least two more acidic residues present between positions -5 and +5 of the sulfated tyrosine occur frequently. Secondly, the presence of turn-inducing amino acids within positions -7 to -2 and +1 to +7 of the tyrosine sulfate residues seem to form a favorable secondary structure for the recognition of substrate proteins by TPST. Finally, no identified tyrosine sulfation site contains a PTM causing steric hindrance like disulfide bonds or N-glycosylation near the tyrosinesulfate residue (Hortin et al., 1986, Huttner, 1987, Niehrs and Huttner, 1990, Niehrs et al., 1990). Later, data from site-directed mutagenesis of human progastrin *in vivo* (Bundgaard et al., 1997) show that basic residues around sulfation site are allowed, though not in position -1.

Frequency. A software tool called Sulfinator for prediction of tyrosine sulfation sites in protein sequences is accessible on the ExPASy server at the URL http://www.expasy.org/tools/sulfinator/ (Monigatti et al., 2002). Scanning with Sulfinator of proteins from various species that according to SWISS-PROT pass through the secretory pathway suggest that one third of

proteins that enter the secretory pathway may contain on average two tyrosine sulfation sites per protein (Monigatti et al., 2002). Another estimation is that 7% of mammalian proteins are tyrosine sulfated (Moore, 2003). According to an *in vivo* labeling study of *Drosophila melanogaster* with inorganic $^{35}SO_4$ as much as 1% of the tyrosine residues of the proteins in an organism can be sulfated (Baeuerle and Huttner, 1985).

Regulation of tyrosine O-sulfation. The regulation of tyrosine *O*-sulfation is not known. The fact that tyrosine sulfation is poorly reversible or even irreversible *in vivo* and *in vitro* suggests that tyrosine *O*-sulfation is not modulated by the sulfatases (Dodgson et al., 1959, Dodgson et al., 1961, Jones et al., 1963, Tallan et al., 1955). Sardinello and co-workers determined by a genomic approach the complete catalog of human sulfatases, which comprises 17 members, but no extracellular sulfotyrosylprotein sulfatase was identified (Sardiello et al., 2005). Tyrosine phosphorylation, which is chemically and structurally a close relative PTM to tyrosine sulfation, is mediated by a rich array of kinases and phosphatases and is involved in multiple signaling and regulatory functions in the cells (Craven et al., 2003, Wang et al., 2003). The small number of TPSTs and the apparent absence of sulfotyrosylprotein sulfatase suggest that protein TPST isoforms are expressed in a cell-specific manner (Bundgaard et al., 1997). However, evidence for transcriptional regulation of the TPST-1 and TPST-2 genes is very limited (Moore, 2003).

Effects of tyrosine-sulfation

Known human tyrosine-sulfated proteins include adhesion molecules, G-protein coupled receptors, coagulation factors, serpins, extracellular matrix proteins, hormones, enzymes and others (Moore, 2003). Post-translational tyrosine *O*-sulfation of proteins may affect protein-protein interactions involved in leukocyte adhesion (Fong et al., 2002, Kehoe and Bertozzi, 2000), hemostasis (Leyte et al., 1991, Michnick et al., 1994, Pittman et al., 1994), chemokine signaling (Kehoe and Bertozzi, 2000), intracellular protein transport and secretion (Friederich et al., 1988), prohormone processing (Bundgaard et al., 1995, Huttner, 1987), receptor-ligand binding (Choe et al., 2005, Costagliola et al., 2002, Gao et al., 2003, Wilkins et al., 1995) and it may influence the biological activity (Brand et al., Dorfman et al., 2006, Hortin et al., 1989) and half-life of proteins (Huttner, 1987).

The HIV-1 envelope glycoprotein has been reported to use sulfotyrosines of the chemokine receptor CCR5 to enter cells that express this obligate coreceptor (Farzan et al., 2002). Likewise, the Duffy antigen/receptor for chemokines (DARC) is necessary for entry of *Plasmodium vivax* malaria into maturing red blood cells, and a sulfotyrosine at the DARC amino terminus mediates its association with the *P. vivax* Duffy-binding protein (Choe et al., 2005). It is suggested that sulfotyrosines may be especially adept at binding diverse proteins with high affinity since the sulfate group distinctively modifies the electronic properties of the phenyl ring of the tyrosine, providing abundant, highly polarizable electrons. Therefore, the sulfate group provides some level of specificity but can also accommodate subtly different microenvironments (Choe and Farzan, 2006).

To assess the role of tyrosine sulfation *in vivo*, *Tpst1* and *Tpst2* knock-out mice have been generated by targeted disruption of the *Tpst1* and *Tpst2* genes (Borghei et al., 2006, Ouyang et al., 2002). Disruption of either the *Tpst1* or *Tpst2* gene decreased postnatal growth. Maternal TPST-1 deficiency also reduced the litter size due to fetal loss and increased perinatal mortality. TPST-2 deficient male, but not female mice, were infertile. It seems that protein(s) required for normal male reproductive function must undergo tyrosine *O*-sulfation to function normally and that these proteins can be sulfated *in vivo* in the absence of TPST-1 but not TPST-2. High affinity and specific anti-sulfotyrosine MAbs have recently

been generated and this will facilitate further investigation and identification of tyrosine-sulfated proteins (Hoffhines et al., 2006, Kehoe et al., 2006).

Post-translational modification of pancreatic trypsinogens

The first evidence for sulfation of pancreatic trypsinogen-1 and -2 came from two-dimensional isoelectric focusing/sodium dodecyl sulfate gel electrophoresis. Incorporation of $^{35}SO_4$ into trypsinogen of pancreatic tissue slices was demonstrated by fluorography of tissue homogenates separated by the two-dimensional gel procedure. Results from acid treatment of the homogenates suggested that the sulfate moiety was covalently attached to tyrosine residue (Scheele et al., 1981). Preliminary ESI MS data from Szilagyi and colleagues (Szilagyi et al., 2001) suggest that the modifying group at Tyr154 in trypsinogen-1 is sulfate and not phosphate as based on the crystal structure study of Gaboriaud and colleagues (Gaboriaud et al., 1996). Later, sulfated tyrosine residues from purified trypsinogen isoenzymes, subjected to alkaline hydrolysis, have been identified by thin layer chromatography (Sahin-Tóth et al., 2006). Furthermore, incorporation of $^{35}SO_4$ into human trypsinogen-1 transiently expressed by human embryonic kidney 239T cells was demonstrated. Mutation of Tyr154 to Phe abolished radioactive sulfate incorporation confirming that Tyr154 is the site of sulfation in trypsinogen-1.

When comparing the sulfated pancreatic trypsinogen-1 and its nonsulfated recombinant form, it was found that the sulfated trypsinogen-1 underwent faster autoactivation. This suggests that tyrosine sulfation might enhance intestinal digestive zymogen activation in humans (Sahin-Tóth et al., 2006). The amidolytic and esterolytic activity of modified and non-modified trypsin-1 are essentially identical, but sulfated trypsin-1 is slightly better inhibited by PSTI (Szilagyi et al., 2001). The finding that mRNA expression of the TPST-2 isoform is drastically higher in the pancreas than in any other tissues examined (Ouyang and Moore, 1998) is thought to explain the high stoichiometry of human pancreatic trypsinogen-1 and -2 sulfation.

Aims of the present study

Tumor-associated trypsin inhibitor (TATI) has been isolated from urine of an ovarian cancer patient (Stenman et al., 1982) and shown to be identical to pancreatic secretory trypsin inhibitor (PSTI) (Huhtala et al., 1982). In search for a target protease for TATI, two trypsinogen isoenzymes were characterized in cyst fluid of mucinous ovarian tumors (Koivunen et al., 1989). The N-terminal amino acid sequences of these tumor-associated isoenzymes corresponded to those of pancreatic trypsinogen-1 and -2 respectively. However, the isoenzymes had different specificities for *p*-nitroanilide substrates, responded differently to various protease inhibitors and had different isoelectric points from those of trypsinogen-1 and -2. Therefore, they were named tumor-associated trypsinogen-1 and trypsinogen-2 (TAT-1 and TAT-2) (Koivunen et al., 1989).

The first aim of the present study was the preparation of specific monoclonal antibodies to trypsinogen isoenzymes, the development of quantitative immunoassays for trypsinogen-1 and -2, and the purification, identification and characterization of pancreatic and tumor-associated trypsinogen isoenzymes by various chromatographic and immunologic techniques.

There have been contradictory reports about post-translational modification of pancreatic trypsinogen-1. The molecular weight of human pancreatic trypsinogen-1 has been determined by mass spectrometry to be 80 Da higher than the theoretical mass deduced from the polypeptide sequence (Gaboriaud et al., 1996, Szilagyi et al., 2001). Based on the x-ray electron density map, the observed mass difference was attributed to phosphorylation at tyrosine residue 154 (Gaboriaud et al., 1996). However, incorporation of $^{35}SO_4$ to trypsinogen-1 and -2 in pancreatic tissue culture (Scheele et al., 1981) on one hand, and alkaline hydrolysis of purified trypsinogens and subsequent separation of modified tyrosine residues by thin layer chromatography on the other hand, has revealed that both human trypsinogen-1 and -2 contain tyrosine sulfate (Sahin-Tóth et al., 2006). Furthermore, trypsinogen-1 expressed in human embryonic kidney 293T cells has been shown to incorporate $^{35}SO_4$ into the secreted trypsinogen and mutation of Tyr154 to Phe was shown to abolish the incorporation (Sahin-Tóth et al., 2006).

The second aim of this study was to characterize by mass spectrometry the chemical modification underlying the observed differences in isoelectric point, substrate binding and inhibitor specificity between pancreatic and tumor-associated trypinogen-1 and -2, respectively.

Materials and methods

The materials and methods are described in detail in the original papers (I-IV).

Samples, patients and cell lines (I-IV)

The ethical committee of Helsinki University Central Hospital, Finland, has approved the use of human samples in this study. Cyst fluid of ovarian tumors was obtained in connection with surgical removal of the tumors. Pancreatic fluid was collected by duodenal catheterization of patients who were examined because of biliary or pancreatic diseases. Benzamidine and aprotinin were added to the pancreatic fluid to a final concentration of 10 mmol/L and 10 µg/L, respectively. Serum samples from patients with pancreatitis and patients who had undergone total pancreatoduodenectomy were kindly provided by Dr. Tom Schröder, Helsinki University Central Hospital, Finland. Serum samples from healthy individuals were collected from the laboratory staff and from women with benign cysts, infertility or pregnancy, and were used to calculate the reference range for trypsinogen-1 and -2. Preovulatory follicular fluid was obtained from patients participating in an *in vitro* fertilization. All samples were stored aliquoted at -20°C or -80°C. The colon adenocarcinoma cell line, COLO 205, was from American Type Culture Collections, and was cultured according to the guidelines provided.

Monoclonal antibodies (I)

Monoclonal antibodies, or MAbs, were obtained by immunizing BALB/c mice (from the Zentralinstitut für Versuchstiersucht, Hannover, Germany) intraperitoneally with 50 µg of TAT containing both isoenzymes emulsified in Freund's complete (first injection) or incomplete adjuvant three times at 2-week intervals. A booster of 10 µg TAT in saline solution was given intravenously four days before fusion. The fusion was performed as described by Köhler and Milstein (Köhler and Milstein, 1975). Antibodies produced by the hybridomas were screened by a TR-IFMA. Hybridomas secreting MAb to trypsinogen-isoenzymes were selected, cloned and expanded. Three MAbs designated 2F3, 3E8 and 6D11 reacted predominantly with trypsinogen-1 and two MAbs, 14D4 and 14F10, reacted with trypsinogen-2.

For the production of large amounts of MAbs in ascites fluid BALB/c mice were primed intraperitoneally with 0.5 mL pristane (96% 2,6,10,14-tetramethylpentadecane, Aldrich-Chemie) one week before the injection of 0.4 – 1 x 10^6 hybridoma cells (Hoogenraad and Wraight, 1986). Ascites fluid was centrifuged and the immunoglobulin fraction from ascites fluid was precipitated with Na_2SO_4 at final concentration of 180 g/L. After washing the precipitate twice it was dissolved in Na_2CO_3 (100 mmol/L, pH 9.0) and stored frozen at -20°C. Alternatively, antibody-producing hybridomas were cultured in INTEGRA CL 1000 flasks (Integra Biosciences), and the MAbs were purified from the culture supernatant by protein G or protein A affinity chromatography (MAbTrap™ or Protein G Sepharose 4 fast flow from GE Healthcare Bio-Sciences or PROSEP-A from Millipore) and MAbs were eluted according to the instructions of the respective manufacturer.

The subclass of the MAbs was determined by immunodiffusion (Ouchterlony, 1958) with specific antibodies from Nordic Immunological Laboratories. All MAbs were of the immunoglobulin G1 isotype.

Time-resolved immunofluorometric assays (I)

MAb production by hybridoma cells was detected by a sandwich TR-IFMA employing

polyclonal rabbit antiserum to human pancreatic trypsin (Koivunen et al., 1989) immobilized onto microtiter wells, to bind partially purified trypsinogen-1 or -2 to the immobilized antibody. The trypsinogen isoenzyme reactive MAbs were detected by rabbit anti-mouse immunoglobulins (Dako) labeled with europium (Eu).

Two TR-IFMAs were developed to recognize trypsinogen-1 and -2, respectively. Each assay is based on the combination of two specific antibodies, catcher antibody immobilized onto microtiter wells and tracer antibody labeled with Eu chelate.

Catcher MAbs were immobilized onto 200 µL polystyrene microtiter wells by incubating 200 µl corresponding to 2 µg of MAb in Na_2CO_3 (100 mmol/L, pH 9.0) overnight at +4°C. To block non-specific adsorption 1% bovine serum albumin in TBS (50 mmol/L Tris-HCl buffer, pH 7.4 containing 9 g/L NaCl and 0.5 g/L NaN_3) was added to the wells and left overnight at +4°C. The BSA-solution was then discarded and the wells were stored in a moist atmosphere at +4°C.

Tracer MAbs were labeled with isothiocyanato-phenyldiethylenetriamine-N^1,N^2,N^3,N^4-tetraacetate chelated with europium(III) (Hemmilä et al., 1984) with a 100-fold molar excess of the chelate. After incubation overnight at +4°C unbound chelate was separated from the labeled MAb by gel chromatography on a 1 x 15 cm column of Sephacryl S200 HR (Pharmacia Biotech) using TBS as eluent. Further purification was achieved by hydrophobic interaction chromatography on a 2 mL phenyl-Sepharose column (Pharmacia, Uppsala, Sweden) equilibrated with TBS. After application of the labeled MAb, the column was eluted with 10 mL of TBS at hydrostatic pressure. The nonadsorbed fraction was collected, stored at +4°C and used as tracer MAb in the TR-IFMA.

All possible combinations of MAbs were tested as catcher and tracer for the optimal assay for trypsinogen-1 and -2. For assay of trypsinogen-1, combinations of MAbs designated 6D11 as catcher and either 2F3 or 3E8 as the tracer were selected. For trypsinogen-2 MAbs 14F10 and 14D4 have been used as either catcher or tracer.

TR-IFMA for trypsinogen-1 and -2 is performed as described (I). Briefly, 25 µL of standard or sample along with 200 µL buffer was incubated in the MAb-coated wells for one hour at room temperature with constant shaking. The wells were washed and 50 ng of Eu-labeled MAb in 200 µL was added and incubated for 30 minutes as above. After washing the wells 200 µL of enhancement solution was added and the mixture was shaken for 5 minutes. Fluorescence was then measured for one second per well in an LKB 1230 Arcus Fluorometer.

TR-IFMA for trypsinogen-1 was calibrated by using PMSF-inhibited pancreatic trypsin-1 covering the concentration range 0.24 to 250 µg/L. Today, TAT-1 recombinant protein is used as standard. TR-IFMA for trypsinogen-2 was calibrated by using the zymogen form of TAT-2 purified from culture medium of COLO 205 cells. The standard curve covered the range 0.97 to 495 µg/L. As assay calibrators we use secondary standards diluted from ovarian cyst fluid.

Sensitivity of the TR-IFMAs for trypsinogen-1 and -2 assay, respectively, was calculated from the mean fluorescence signal of a zero sample (assay buffer, n=20) plus two standard deviations. The intra- and inter-assay variation of the assays was calculated from the repetitive results of control samples (mucinous ovarian cyst fluids diluted in assay buffer) stored at -20°C. The cross-reaction of each isoenzyme in the assay for the other isoenzyme was confirmed by assaying trypsinogen-1 and -2 separated by anion exchange chromatography.

Radioimmunoassays (I and II)

RIA kits for the determination of trypsin-like

immunoreactivity were obtained from CIS and were used according to the instructions of the manufacturer. The assay employs rabbit antiserum to human trypsin-1, ^{125}I labeled trypsin-1 as tracer, and is calibrated with trypsin-1 purified by Trasylol (aprotinin)-affinity chromatography. The serum sample volume used in the assay was 100 µL.

RIA of TATI was performed employing a polyclonal rabbit antiserum and ^{125}I labeled TATI as described (Stenman et al., 1982). Concentration of TATI in normal serum was reported to be 5 to 20 µg/L.

Characterization of trypsinogens by gel filtration chromatography (I and II)

Serum samples (0.5 mL) were applied to a 1 x 35 cm Sephacryl S-200 column and eluted with TBS at a flow rate of 20 mL/hr at +4°C. One mL fractions were collected and 100 µL of a solution that contained 50 g bovine serum albumin, 5 g bovine immunoglobulin, 10 mg aprotinin and 1 g Tween-40 per liter was added to each fraction. Immunoreactivity of the fractions was determined by TR-IFMA for trypsinogen-1 and -2 with a sample volume of 200 µL.

Gel filtration of hyperstimulated follicular fluid and filtered ovarian cyst fluid was performed on Superose 12 column (Pharmacia) in TBS at a flow rate of 0.5 mL/minute. The sample volume was 200 µL and fraction size 400 µL.

The gel filtration columns were calibrated with albumin 67 kDa), ovalbumin (43 kDa), soybean trypsin inhibitor (21 kDa) and aprotinin (6 kDa).

Purification of trypsinogen (I, II and IV)

Trypsinogen from mucinous ovarian cyst fluid was purified by a combination of batch-wise anion exchange, immunoaffinity, and reverse-phase (RP) chromatography as described elsewhere (Koivunen et al., 1989). Briefly, cyst fluid was centrifuged and dialyzed against distilled water with a hollow fiber dialyzer. The dialyzed cyst fluid was mixed overnight with a strong anion exchanger, Q Sepharose (Pharmacia Biotech). After washing away unbound material, bound proteins were eluted with 1 mol/L NaCl and 1% isopropanol. Trypsinogen from human pancreatic juice or conditioned media from COLO 205 cells was purified by immunoaffinity chromatography after centrifugation and pH adjustment to 7.5. Before immunoaffinity chromatography, benzamidine, aprotinin and Brij 35 were added to final concentration of 10 mmol/L, 10 mg/L and 0.1%, respectively, to all preparates.

Immunoaffinity columns were produced by coupling MAbs 3E8 for trypsinogen-1 and MAb 14F10 for trypsinogen-2 to CNBr-activated Sepharose 4B (Pharmacia Biotech) according to the manufacturer's instructions. The starting material was pumped at a flow rate of 10 to 30 mL/h through the two immunoaffinity columns connected in tandem. After sample application the columns were separated and washed. The bound fraction was eluted with 0.1% TFA containing 1 mmol/L $CaCl_2$, 10 mmol/L benzamidine and 10 mg/L aprotinin, collected in one mL fractions, neutralized and assayed for trypsinogen immunoreactivity. Fractions containing trypsinogen were further purified by ion exchange or reverse-phase HPLC. All purification steps except HPLC were carried out at +4°C.

Separation of trypsinogen iso-enzymes by anion exchange and RP HPLC (I, III and IV)

Before anion exchange chromatography was performed, serum samples and cell culture media were diluted fivefold, and cyst fluids were diluted 10- to 30-fold with 50 mmol/L Tris-HCl buffer, pH 8,0 (buffer A). Five hundred µL of diluted sample was applied to a Mono Q HR 5/5 or Resource Q anion exchange column (Pharmacia Biotech) equilibrated with buffer A and eluted with a linear gradient (0

to 100% in 40 or 60 minutes, respectively) of buffer A containing 1 mmol/L $CaCl_2$, 0.5 mol/L NaCl, and 0.1% isopropanol. Fractions of 0.5 to 2 mL were collected and assayed for trypsinogen-1 and -2. Trypsinogen isoenzymes isolated by immunoaffinity chromatography were chromatographed in the same manner.

Immunoaffinity purified trypsinogen preparations were further purified by RP HPLC on either 8 x 100 mm µ-Bondapak C18 Radial-PAK or 3.9 x 75 mm Nova-Pak C18 column (Waters), C1 (2.1 x 100 mm) or C4 (3.9 x 20 mm) column. Trypsinogen was eluted with a linear gradient of 0.1% TFA in ACN. A HPLC system from LKB, ÄKTA prime or ÄKTA purifer (Amersham Biosciences) was used.

Activation of trypsinogen isoenzymes (I and II)

Trypsinogen isoenzymes were purified by ion exchange HPLC and divided to three aliquots. Aprotinin was added to a concentration of 70 mg/L to two aliquots to prevent autoactivation. One of these was activated by enteropeptidase (EC 3.4.21.9, Sigma) by incubation at 23°C for 16 hours. The third aliquot was activated in the absence of aprotinin and activation was confirmed by assay of enzyme activity with the synthetic *p*-nitroanilide substrate S-2222 (Kabi) (Koivunen et al., 1989). Immunoreactivity of the trypsin-aprotinin complex was compared with that of the proenzyme (I). Alternatively, trypsinogen was autoactivated in neutral pH at 37°C for 2 hours (II).

Alkylation and digestion of trypsinogens (IV)

Purified trypsinogens were reduced with dithiotreitol and alkylated with 4-vinylpyridine (Aldrich). The alkylated proteins were desalted by RP HPLC. Trypsinogen containing fractions were pooled, dried and subjected to trypsin digestion using 5% w/w sequencing grade trypsin (Promega Ltd). The tryptic peptides were separated by RP HPLC, collected and analyzed by MALDI-TOF mass spectrometry.

For further digestion with chymotrypsin, selected peptides were dried and dissolved in 50 mmol/L Tris-HCl buffer containing 0.6 mol/L urea. Five % (w/w) chymotrypsin (Sigma) was added and digestion was carried out at 37°C overnight. The chymotryptic peptides were separated by RP HPLC and collected as above.

Mass spectrometry (IV)

Matrix-assisted laser desorption ionisation time of flight (MALDI-TOF) MS was performed with a Biflex MALDI-TOF mass spectrometer (Bruker-Daltonics) equipped with a nitrogen laser operating at 337 nm. Before MALDI-TOF analysis, the protein digests were desalted using poros R3 (PerSeptive Biosystems) material. The peptides were eluted directly onto the MALDI sample plate with α-cyano-4-hydroxy-cinnamic acid matrix in 0,1% TFA, 50% ACN. RP HPLC –purified proteolytic peptides were pipetted directly onto a MALDI sample plate with an equal volume of matrix and dried under a gentle stream of warm air. Peptides were analyzed either in the positive ion reflector or linear mode.

Electrospray ionization (ESI) MS analyses of purified trypsinogens and their digests were performed using a Micromass Q-TOF quadrupole/time-of-flight hybrid mass spectrometer (Q-TOF Micro, Waters). For analysis of intact proteins, the trypsinogens were injected into the mass spectrometer via a nanoflow interface with a Hamilton-syringe pump. The digested peptides were injected into the mass spectrometer after fractionation by nanoscale RP-HPLC on the CapLC (Waters) with a 0.075 x 150 mm C18 column (Symmetry C18, 300 Å, 3.5 µm, Waters) that was eluted with a linear gradient of ACN (5-50% in 30 min) in 0.1% formic acid. Flow rate was 0.25 µL/min and the eluent was directly injected into the mass spectrometer. The capillary voltage was 2000 V and the source block temperature 120 ºC. The sampling cone was

operated normally at 45 V, but at in-source dissociation experiments ramped from 30 to 70 V. Tandem mass spectrometric (MSMS) fragmentation spectra of the peptides were acquired by colliding the doubly or triply charged precursor ions with argon collision gas at accelerating voltages of 30-45 V. As sulfotyrosine and phosphotyrosine standard peptides we used DsYMGWMDF (1134.442 Da) from Bachem and YRMKKKDEGSpYT (1584.714 Da) synthetized on a 433A automatic peptide synthetizer (Applied Biosystems).

Data analysis (IV)

Data analysis of intact protein ESI mass spectra was carried out with MassLynx software (Waters) and PAWS proteomic analysis software (ProteoMetrics). Mass spectra collected during the LC-MS separation of digested peptides were exported into ASCII text files using the DataBridge of the MassLynx software (Waters). The text files were imported into the DeCyder MS software (GE Healthcare), where different elution profiles were visualized as two-dimensional graphs and different *m/z* values deconvoluted into molecular masses of 700-6000 Da. The ion counts of all different charge states of the same peptide were taken into account to calculate total intensity of the deconvoluted masses.

N-terminal sequence analysis (IV)

NH_2-terminal sequence analyses were performed by the Edman degradation using a Procise 494A sequencer (Applied Biosystems).

Electrophoresis and immuno-blotting (II and IV)

SDS-PAGE was performed according to Laemmli (Laemmli, 1970) under non-reducing conditions on 20% polyacrylamide Phastgels using the Phastsystem (Pharmacia) and stained with silver according the manufacturer's instructions. For Western blot analysis the proteins were separated on regular 12.5 % acrylamide gels and transferred to a PVDF membrane (Immobilon-P, Millipore). The membrane was incubated with anti-phosphotyrosine antibody (clone 4G10, Upstate) and MAb 14F10 specific for trypsinogen-2. Immunoreactive proteins were detected with enhanced chemiluminescense (Super Signal West Femto Maximum Sensitivity detection kit, Pierce Biotechnology).

Statistical analysis (II)

Student's unpaired *t* test and Mann-Whitney U test were used to estimate the differences between the trypsinogen levels in the various ovarian cyst fluids and serum samples, respectively.

Results

Immunoassays for trypsinogens/ trypsin and concentrations in serum samples (I, III)

The sensitivity of the TR-IFMAs for trypsinogen-1 and -2 was 0.1 and 0.3 µg/L, respectively. The sensitivity could be increased up to eight-fold by increasing the sample volume to 200 µL. It is reported by the manufacturer that the commercial RIA for trypsin determines trypsin-like immunoreactivity, eventually trypsinogen-1, in serum or plasma. The immunoreactivity of trypsin-1 as compared to trypsinogen-1 is reported to be 60%.

For the TR-IRMA for trypsinogen-1 there was no effect of proenzyme activation on the immunoreactivity as studied by activated and aprotinin-inactivated trypsin-1. However, in the TR-IRMA for trypsinogen-2 the immunoreactivity of the activated and aprotinin-inhibited trypsin-2 and of highly purified PMSF-inhibited trypsin-2 was only 57% and 10%, respectively. Comparison of commercial RIA for trypsin and the developed TR-IFMA for trypsinogen-1 revealed good correlation at concentrations higher than 30 µg/L (Figure 2). The correlation was r = 0.83 for control samples (n = 11) and r = 0.90 for pancreatitis samples (n = 20). Despite the reported sensitivity (2 µg/L) and standard curve range (5 to 400 µg/L) RIA discriminated poorly below 30 µg/L. TAT-2 purified from COLO 205 cell culture media by immunoaffinity and ion exchange chromatography was not detected by the RIA. For the TR-IFMAs for trypsinogen isoenzymes the cross-reaction of each isoenzyme in the assay for the other one was confirmed to be less than 1% by assaying trypsinogen-1 and -2 separated by anion exchange chromatography.

The concentration of trypsinogen-1 (median 21 µg/L) was higher than that of trypsinogen-2 (median 17 µg/L) in serum of healthy subjects and patients with extrapancreatic disease. The reference range of this control group for trypsinogen-1 and -2 was calculated to be 5.6 to 69 µg/L and 5.1 to 53 µg/L, respectively. However, in acute pancreatitis the ratio of trypsinogen isoenzymes in serum is reversed: the concentration of trypsinogen-2 is 50-fold higher than in control sera, whereas the difference in trypsinogen-1 concentration is 15-fold (Figure 2). In serum samples from patients who have undergone total pancreatoduodenectomy one of nine contained trypsinogen-1 immunoreactivity (2 µg/L), whereas all samples contained trypsinogen-2 (median 3 µg/L), the mean level being one fifth of that in control sera. The levels of trypsinogen isoenzymes, trypsin-like immunoreactivity and TATI in serum samples, ovarian follicular

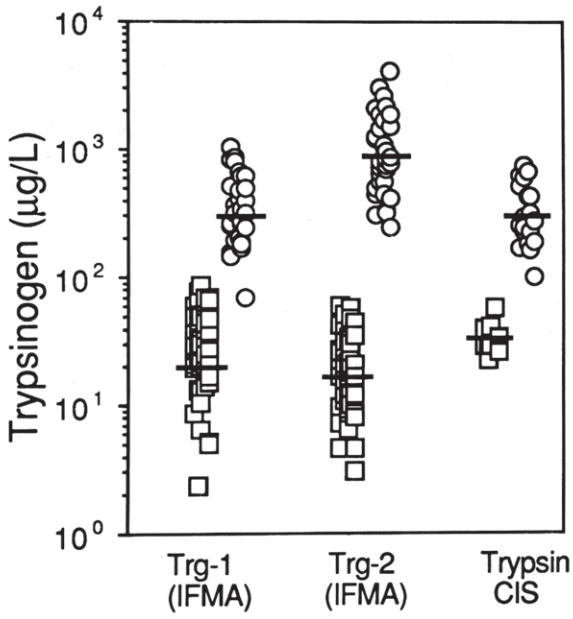


Figure 2. Serum concentrations of trypsinogen-1 (Trg-1) and -2 (Trg-2) measured by TR-IFMA and of trypsin measured by RIA form CIS in sera from healthy subjects (□) and patients with acute pancreatitis (o). Lines indicate median of each group.

Table 4. The levels of immunoreactive trypsinogen-1, -2, trypsin and TATI in human samples.

		n	Trypsinogen-1 (µg/L)		Trypsinogen-2 (µg/L)		Trypsin by RIA (µg/L)		TATI (µg/L)	
			median	range	median	range	median	range	median	range
Serum										
	Control group	144	21	2.3 - 85	17	2.3 - 60	29 (n=11)	23 - 58	11[a]	5 - 20[a]
	Acute pancreatitis	35	313	69 - 1024	813	242 - 3059	275 (n=20)	99 - 740	87[b]	2 - 978[b]
	Pancreatectomy	9	2 (n=1)	- - -	3	2 - 11	<34[c]	- - -	15[c]	7 - 148[c]
Ovarian follicular fluid		16	22	6 - 37	15	6 - 26	n.d	n.d	n.d	n.d.
Ovarian cyst fluid										
Mucinous	Benign	22	16	1 - 206	84	11 - 10 400	n.d	n.d	2 360	7 - 9 840
	Borderline or malignant	5	42	2 - 1260	2640	6 - 31200	n.d	n.d	6 640	1880 - 1 000
Serous	Benign	6	8	1 - 31	18	8 - 44	n.d	n.d	13	3 - 18
	Borderline or malignant	5	86	18 - 127	345	201 - 1790	n.d	n.d	15	11 - 1200
Other	Benign	5	17	2 - 25	32	5 - 95	n.d	n.d	10	3 - 14
	Malignant	7	31	3 - 111	62	15 - 563	n.d	n.d	69	6 - 240

n.d. not determined, [a]Stenman et al., 1982, [b]Haglund et al., 1986: twenty of 25 patients had acute and five chronic pancreatitis, [c]Halila et al., 1985

fluids and ovarian cyst fluids are summarized in Table 4.

TAT and TATI concentrations in ovarian tumor cyst fluids (II)

In hyperstimulated ovarian follicular fluid the median levels of TAT-1 (22 µg/L) and -2 (15 µg/L) corresponded to those in normal serum, TAT-1 being the main isoenzyme. However, in ovarian cyst fluids TAT-2 was the predominant form and its concentrations were significantly higher than those in control sera or ovarian follicular fluids. TAT-2 concentration was higher in mucinous than in serous cyst fluid, and especially in mucinous cyst fluids the concentration of TAT-2 was higher in borderline or malignant (median 2 640 µg/L) than benign cases (median 84 µg/L). Also in serous and other types of borderline and malignant ovarian carcinomas the TAT-2 concentration was higher in the benign cases (Figure 3).

Very high concentrations of TATI occurred in mucinous ovarian cyst fluids, both in benign and malignant ones (Table 4). In contrast, TATI concentrations in serous cyst fluids (3 to 21 µg/L) were similar to those in normal serum (Stenman et al., 1982) except for one malignant adenocarcinoma. TATI levels were not elevated in other benign ovarian tumors, but four malignant ones exhibited high levels (69 to 240 µg/L).

Characterization of trypsinogen immunoreactivity by gel filtration (I, II)

Immunoreactive trypsinogen in serum from healthy individuals and patients with acute pancreatitis, ovarian follicle fluid and ovarian cyst fluid was characterized by gel filtration. The elution pattern of these samples was identical. Trypsinogen-1 and -2 immunoreactivity eluted with molecular masses about 25 kDa and 28 kDa, respectively, indicating that it consisted of the zymogen form, and not of trypsin. In addition, a minor peak with higher molecular mass could be seen in the assay for trypsinogen-2. This peak was later shown to represent trypsinogen-2 – α_1-protease inhibitor complex (Hedström et al., 1994).

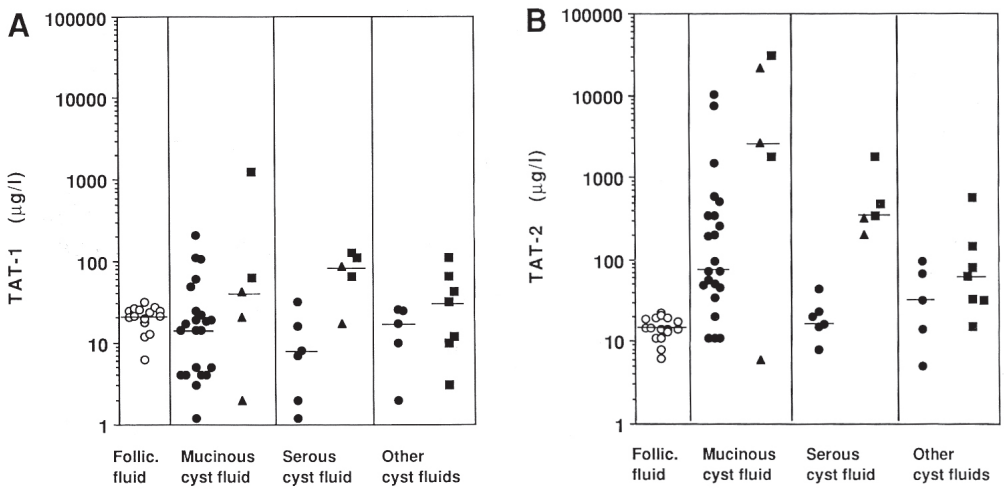


Figure 3. Concentrations of TAT-1 (A) and TAT-2 (B) in normal follicular (Follic.) fluid (O) and cyst fluid from benign (●), borderline (▲), and malignant (■) ovarian tumors. Bars indicate median of each group.

Trypsinogens in anion exchange chromatography (I, III)

Trypsinogen-1 and -2 were separated by anion exchange HPLC from serum from healthy controls, patients with acute pancreatitis and pancreatoduodenectomy, cyst fluid of mucinous ovarian cancer and conditioned culture medium from COLO 205 cells. Under identical elution conditions two immunoreactive forms of each isoenzyme could be seen in these samples. In the serum samples the main trypsinogen-2 peak eluted in fractions 53 to 56 (Figure 4AC) and the minor peak in fractions 40 to 43. The less acidic, earlier eluting form of trypsinogen-2 comprised 10 to 20% of total immunoreactivity in normal serum and less than 10% of total immunoreactivity in pancreatitis serum. The latter, less acidic one corresponded to the main form of trypsinogen-2 in mucinous ovarian cyst fluid and conditioned medium of COLO 205 cells. The more acidic, main form of

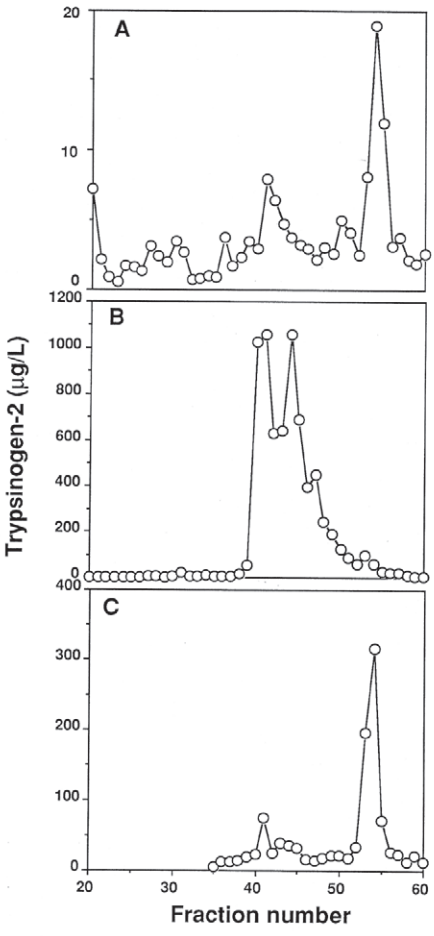


Figure 4. Comparison of elution patterns of trypsinogen-2 isoenzymes in (A) serum from a patient who had undergone pancreatectomy, (B) mucinous ovarian cyst fluid, and (C) serum from a patient with pancreatitis, as measured after anion exchange chromatography.

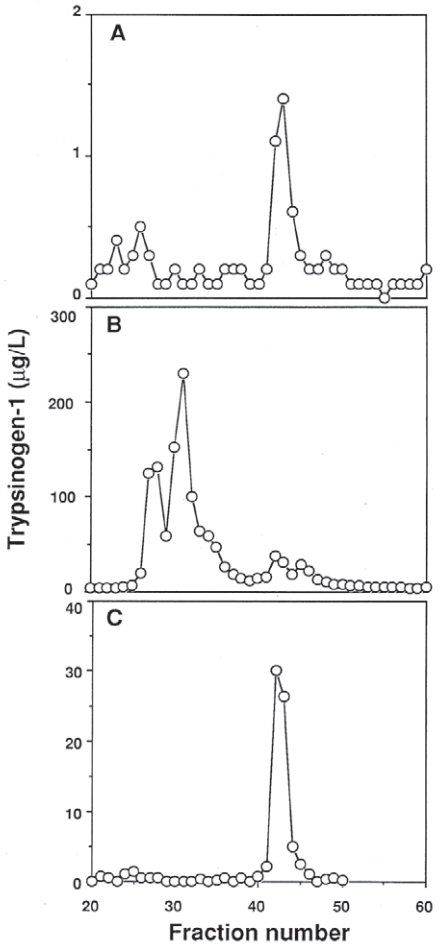


Figure 5. Comparison of elution patterns of trypsinogen-1 isoenzymes in (A) serum from a patient who had undergone pancreatectomy, (B) mucinous ovarian cyst fluid, and (C) serum from a patient with pancreatitis, as measured after anion exchange chromatography under equal conditions as in figure 4.

trypsinogen-2 in serum could not be detected in cyst fluid of ovarian cancer (Figure 4B).

The serum samples contained one main trypsinogen-1 peak eluting in fractions 41 to 47, and a minor, less acidic peak comprising less than 5% of the total immunoreactivity in fractions 26 to 33 (Figure 5AC). In mucinous ovarian cyst fluid and conditioned culture medium from COLO 205 cells the latter form was predominant, whereas the later eluting, more acidic trypsinogen-1 form comprised only 5% to 10% of the total immunoreactivity in ovarian cyst fluid (Figure 5B).

Purification of trypsinogen by reverse-phase HPLC (II, IV)

Immunoaffinity purified trypsinogen isoenzymes from mucinous ovarian cyst fluid, pancreatic juice and conditioned medium from COLO 205 cells were further purified by RP HPLC. To confirm the immunoreactivity measured by TR-IFMA, trypsinogen-2 was autoactivated and purified by RP HPLC. The fractions containing trypsin activity were analyzed by SDS-PAGE and revealed a major band of 27 kDa corresponding to trypsin-2.

MS-analysis of trypsin and trypsinogen isoenzymes (IV)

Purified trypsin and trypsinogen isoenzymes were analyzed by ESI-MS and the intact proteins gave clear *m/z* envelope. The trypsins appeared in the mass spectra mainly in charge states from $[M+11H]^{11+}$ to $[M+16H]^{16+}$. Deconvolution of these spectra showed that the mass of trypsin-1 was 24185.0 Da, as earlier reported (Gaboriaud et al., 1996) and

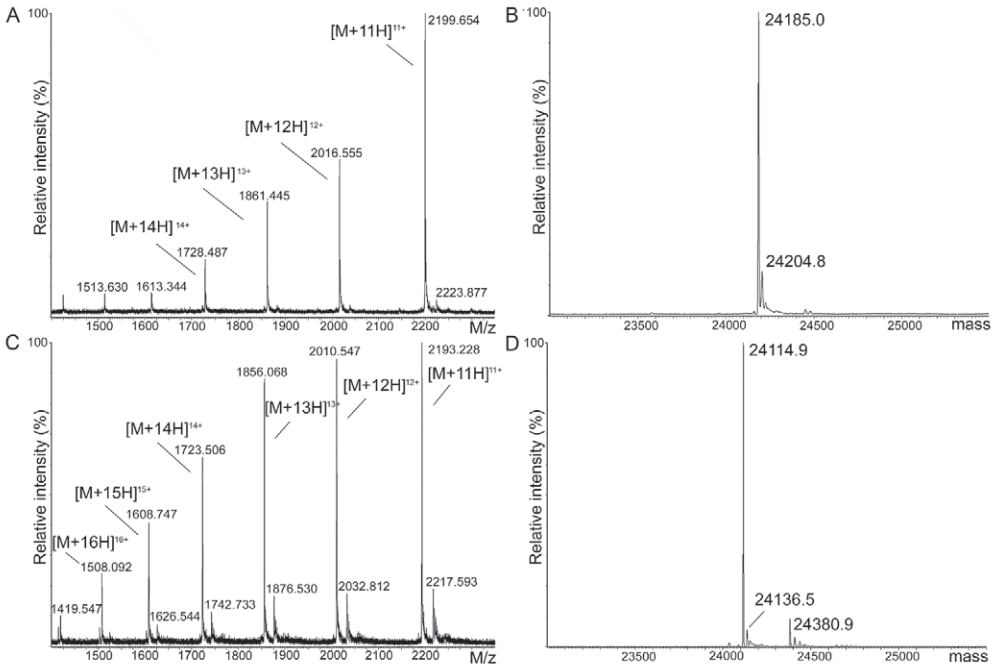


Figure 6. Mass spectrometric analyses of trypsin isoenzymes purified from pancreatic juice. ESI mass spectra of trypsin-1 (A) and -2 (C) reveal peaks mainly of $[M+11H]^{11+}$ to $[M+16H]^{16+}$ of the molecule as indicated. The deconvoluted spectra show that the mass of trypsin-1 is 24185.0 (B) and that of trypsin-2 is 24114.9 (D).

that of trypsin-2 was 24114.9 Da (Figure 6). Both of the masses are 80 Da higher than the theoretical masses calculated for trypsin-1 (24104.2 Da) and -2 (24033.9 Da) with all the putative disulfide bridges present and the propeptide cleaved as a result of activation.

Similar results and mass addition of 80 Da were obtained with the proenzymes: the masses were 25086.0 Da and 25016.0 Da for trypsinogen-1 and -2, respectively. However, the deconvoluted mass of TAT-2 purified from conditioned medium of COLO 205 cells was 24937.0 Da, which corresponds to the theoretical mass calculated for trypsinogen-2 (24937.8 Da) with all putative disulfide bridges and the propeptide present (Figure 7).

Identification of the tryptic peptide with 80 Da mass addition (IV)

Highly purified pancreatic trypsinogen isoenzymes were alkylated and digested with trypsin. The digests were analyzed by MALDI-TOF and LC-MS. Two-dimensional visualization of the LC-MS spectra revealed a tryptic peptide with the mass of 3598.618 as $[M+4H]^{4+}$ with *m/z* of 900.654 comprising trypsinogen-1 amino acids 139 to 170 with an 80 Da mass addition (Figure 8A). Similarly, a peptide with the mass off 5923.850 was observed as $[M+5H]^{5+}$ with *m/z* of 1185.770 corresponding trypsinogen-2 amino acids 123 to 178 with an 80 Da mass addition (Figure 8B).

Identification of Tyr154 sulfation in trypsinogen-1 and -2 (IV)

In-source dissociation. The modification in the pancreatic trypsinogen peptides was characterized by ESI-MS using increasing cone voltages to induce in-source dissociation. The peptides comprising amino acids 139 to 170 in trypsinogen-1, 123 to 178 in trypsinogen -2, respectively, and standard peptides containing phosphotyrosine and sulfotyrosine were analyzed under equal conditions. No loss of 80 Da was observed from the phosphotyrosine standard peptide at sampling cone voltage 30 V, 45 V or 70 V. In contrast, 87% of the sulfotyrosine containing peptide appeared in a non-sulfated form at low cone voltage (30 V) and the lability of the sulfogroup increased at higher cone voltages of 45 V and 70 V (Figure 9). The tryptic peptides of pancreatic trypsinogen-1 and -2 showed similar loss of 80 Da as the standard sulfotyrosine peptide when the cone voltage was ramped from 30 V to 45 V and 70 V. At cone voltage 30 V some

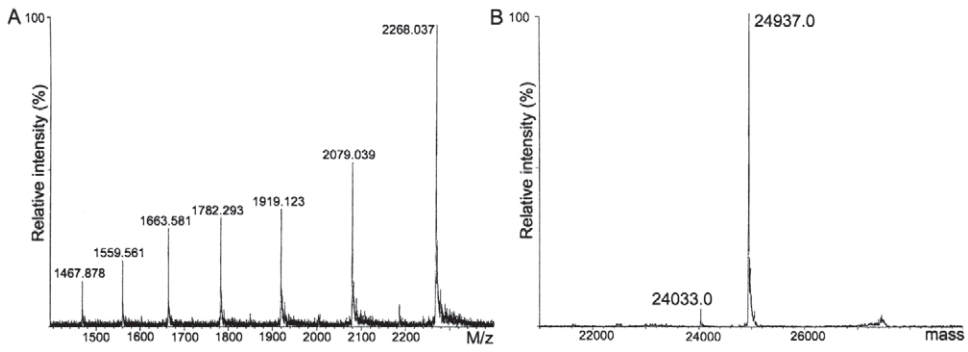
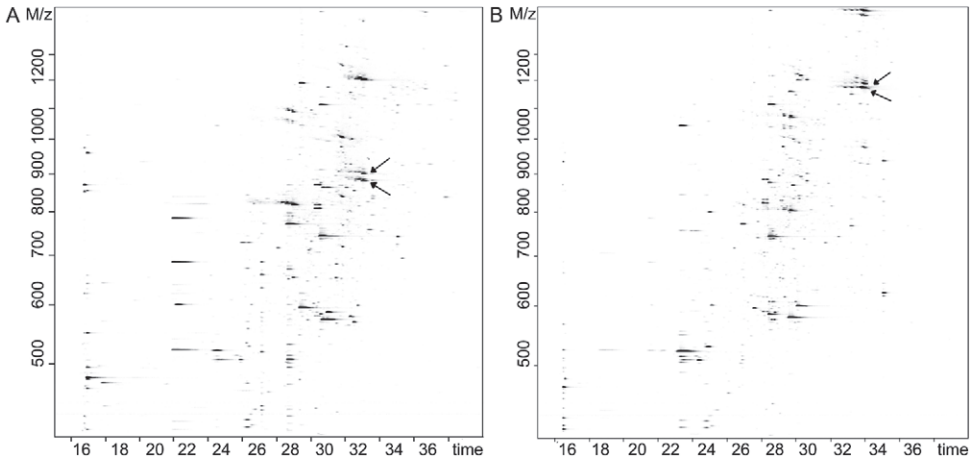


Figure 7. Mass spectrometric analysis of tumor-associated trypsinogen-2. ESI mass spectra of trypsinogen-2 purified from the medium of a colon carcinoma cell line is shown in panel A. The deconvoluted spectra indicates the mass of tumor-associated trypsinogen-2 to be 24 937.0 (B), which corresponds to the theoretical mass of the peptide without post-translational modifications.



C

Peptide	Position (amino acids)	Theoretical m (Da)	Observed m (Da)
P1	16-23	921,371	
P2[a]	24-66	5022,299	5022,359
P5	73-92	2224,112	2224,135
P7	96-101	814,372	
P9	103-112	1173,643	1173,647
P12	123-138	1539,851	1539,871
P13[a]	139-170	3518,648	3518,659
P13[a] + 80		3598,648	3598,618
P14[a]	171-178	958,422	958,426
P15[a]	179-193	1706,816	1706,842
P16[a]	194-223	3265,438	3265,455
P17	224-231	905,497	
P18	232-237	784,412	784,425

[a]ethylpyridyl-modified cysteines

D

Peptide	Position (amino acids)	Theoretical m (Da)	Observed m (Da)
P1	16-23	921,371	
P2[a]	24-66	4994,329	4994,343
P5	73-92	2224,112	2224,094
P9	103-112	1156,670	1156,665
P10	113-122	1042,577	1042,577
P11[a]	123-178	5843,786	5843,749
P11[a] + 80		5923,786	5923,850
P12[a]	179-193	1733,827	1733,840
P13[a]	194-223	3207,439	3207,411
P14	224-231	933,503	933,504
P15	232-240	1198,602	1198,609

[a]ethylpyridyl-modified cysteines

Figure 8. Tryptic peptides of trypsinogen-1 and -2. Panels A and B show LC-MS separation of pancreatic trypsinogen-1 and -2 tryptic peptides, respectively. Masses of tryptic peptides derived from 4-vinylpyridine-alkylated trypsinogen-1 and -2 are shown in panels C and D, respectively. The observed masses are mean values calculated on the basis of the many observed *m/z* values and corresponding charge states. Peptide sequences assigned to each observed mass is indicated by amino acid positions and the theoretical mass of the assigned sequence are shown. The $[M+4H]^{4+}$ of trypsinogen-1 peptide and $[M+5H]^{5+}$ of trypsinogen-2 peptide containing a 80 Da mass addition are visualized with upper arrows and the same charge states of the same peptides without the mass addition are visualized with lower arrows in panels A and B, respectively.

loss of 80 Da could be detected and at 70 V more than 82% of the peptides lost 80 Da from their mass.

Collision-induced dissociation. The sulfation of the trypsinogen peptides was further supported by the results obtained from collision-induced dissociation (CID) in LC-MSMS. Under CID conditions no loss of HPO_3 moiety from the standard phosphopeptide was observed (Figure 10) as earlier also reported (Nemeth-Cawley et al., 2001). Our CID analysis on mass modified trypsinogen-1 peptide (amino acids 139 to 170) and trypsinogen-2 peptide (amino acids 147 to 178 produced by in-source dissociation from peptide comprising amino acids 123 to 178) reveal an unmodified tyrosine at position 154 indicating loss of the SO_3 moiety before

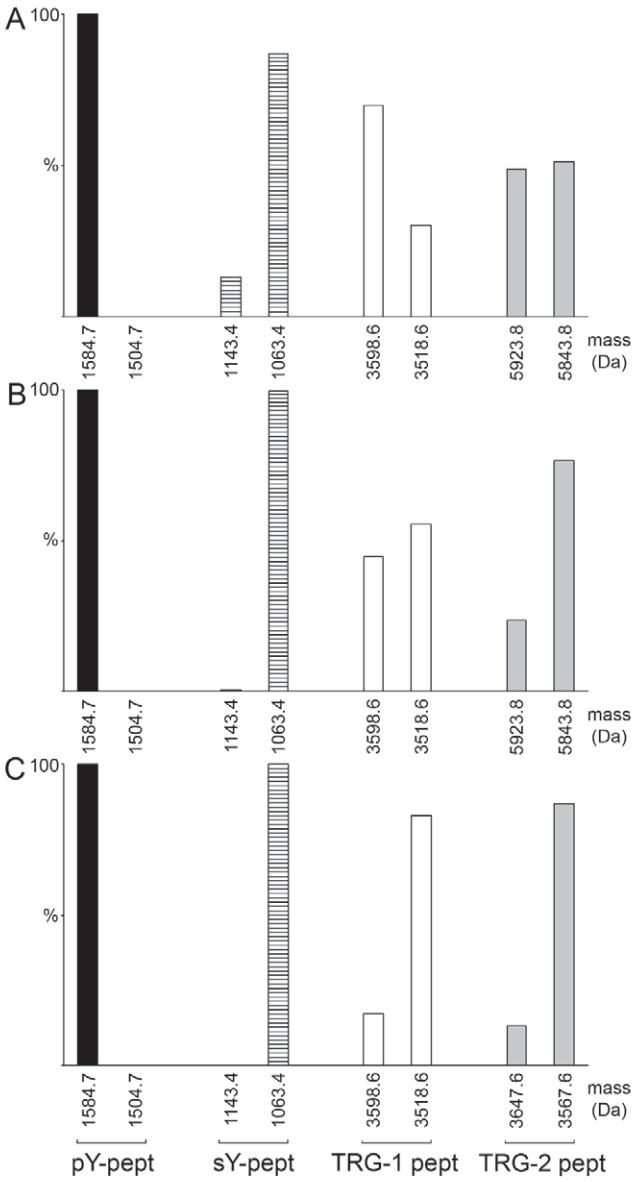


Figure 9. In-source dissociation of the sulfate group of the modified trypsinogen-1 and -2 peptides. Phosphotyrosine (black, pY-pept) and sulfotyrosine (striped, sY-pept) containing peptides together with trypsinogen-1 peptide comprising amino acids 139-170 (white, TRG-1 pept) and trypsinogen-2 peptide of amino acids 123-178 (grey, TRG-2 pept) were analyzed by LC-MS using cone voltages of 30 V (A), 45 V (B) and 70 V (C). The proportion of sulfated peptide decreases with increasing voltage. At 70 V a peptide bond in the trypsinogen-2 peptide is broken resulting in a peptide consisting of amino acids 147-178, which is visualized in panel C. Different charge states of the same peptides were detected, their intensities integrated and the total intensity of the deconvoluted masses shown were calculated. The proportions of peptides with (left) and without (right) the 80 Da mass modification are indicated.

the backbone fragmentation (Figure 10).

Immunoblotting and Edman degradation. In immunoblotting experiment with anti-phosphotyrosine antibody no signal of pancreatic trypsinogen-2 was detected. Furthermore, fifteen cycles of N-terminal sequence analysis by Edman degradation of the purified chymotryptic peptide of pancreatic trypsinogen-2 comprising amino acids 149 to 178 gave a sequence SSGADYPDELQCLDA. The signal of the phenylthiohydantoin (PTH) derivative of tyrosine in position six (Tyr154) corresponds to the signals of the amino acid derivatives obtained from the other positions.

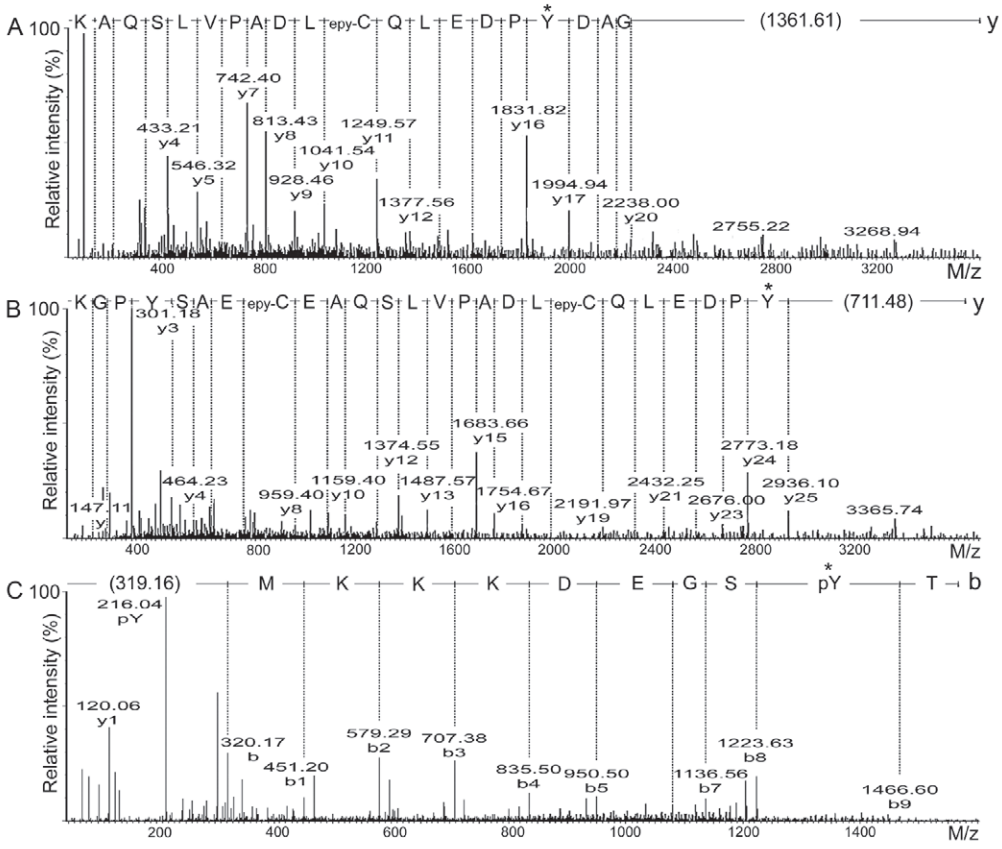


Figure 10. MSMS fragmentation spectra of tryptic peptides containing the modified amino acid (Tyr154) in pancreatic trypsinogen isoenzymes. Panel A shows the MSMS fragmentation spectra of the triply charged precursor ion of *m/z* 1200.54 representing a sulfated tryptic peptide (mass 3598.60) comprising amino acids 139-170 in trypsinogen-1. The MSMS spectra of the triply charged precursor ion of *m/z* 1216.55 (mass 3646.63) is likely to represent in-source dissociated fragment of the tryptic peptide of trypsinogen-2 containing amino acids 147-178 (panel B). The peptide sequences derived from fragmented y-ion series are annotated on the spectra. Cysteine alkylated with an ethylpyridyl group is abbreviated epy-C. Panel C shows the fragmentation spectra of a phosphotyrosine-containing synthetic peptide (mass 1584.71), in which the fragmentation derived b-ion series is annotated on the spectra. The sulfated tyrosine residues of the precursor ions are indicated by asterisks.

Discussion

TR-IFMAs for trypsinogen-1 and -2

The first aim of the present study was to produce monoclonal antibodies to trypsinogen isoenzymes identified in cyst fluid of ovarian cancer patients. We were able to produce several high affinity MAbs by immunization of mice with a preparation containing both TAT-1 and -2 isoenzymes. These MAbs were suitable for the development of immunofluorometric assays, and for purification and detection of trypsinogen isoenzymes by various immunologic techniques.

We developed highly sensitive and specific TR-IFMAs for trypsinogen-1 and -2 based on microtiterplate technology. The TR-IFMAs are sandwich-type assays with, by design, specificity for two epitopes on a molecule. The catcher MAb is immobilized onto microtiterplate wells in high concentration resulting in high sensitivity, wide analytical range and high recovery (Alfthan, 1986). The tracer MAb is labeled with lanthanide europium. Five to fifteen Eu molecules can be incorporated into an antibody molecule (Soini and Kojola, 1983) and high tracer concentration contributes to assay sensitivity (Alfthan, 1986). The lanthanide chelates have long Stokes' shift, narrow emission peaks and exceptional decay times, which allows easy and efficient background discrimination (Hemmilä and Laitala, 2005). This results in low background reading and high sensitivity in immunoassays when measuring time-resolved fluorescence (Hemmilä et al., 1984, Soini and Kojola, 1983, Soini et al., 1990). The shelf-life of lanthanide-labeled tracer is long (Alfthan, 1986). These properties of luminescent lanthanide chelates make them superior as compared to other absorptive, fluorescent or radioactive probes in immunoassays. The TR-IFMAs have good precision due to technical convenience and robustness.

High specificity in the developed TR-IFMAs was achieved by combination of two MAbs with preferential reactivity for one isoenzyme. We compared our TR-IFMA for trypsinogen-1 with a commercial radioimmunoassay for trypsin and found that the assays correlated well at concentrations above 30 µg/L, but the RIA discriminated poorly at low concentrations. The detection limits of the TR-IFMAs for trypsinogen-1 and -2 were 0.1 and 0.3 µg/L, respectively.

The immunoreactivity of DFP-inactivated trypsin-1 has been shown to be different from that of the proenzyme in some (Borgström and Ohlsson, 1976) but not in all (Lafont et al., 1995) immunoassays. Anyhow, trypsinogen-1 and trypsin-1 inhibited with aprotinin or PMSF reacted equally in our assay for trypsinogen-1. Thus, we could use trypsin-1 inhibited with PMSF as standard in the assay for trypsinogen-1, as purified trypsinogen-1 was not available in sufficient amounts. On the other hand, trypsin-2 inhibited with aprotinin or PMSF had clearly reduced immunoreactivity in the assay for trypsinogen-2. This could be the result of autodegradation of the highly purified and concentrated trypsin-2 preparation, as trypsin-2 has been shown to be prone to autolysis (Mallory and Travis, 1973, Rinderknecht and Geokas, 1972). It appears to be important to use the proenzyme as a standard in the TR-IFMA for trypsinogen-2.

Immunoreactive trypsinogen-1 and -2 in serum samples

Healthy subjects. Trypsinogens are mainly produced by the exocrine pancreas and secreted at high concentrations into pancreatic fluid (Rinderknecht and Geokas, 1972). A small portion of trypsinogens and active trypsins escapes into the circulation, where trypsinogen remains free but active trypsins

are rapidly inactivated mainly by α_2M and API (Borgström and Ohlsson, 1978). Trypsinogen-1 concentrations in serum measured by us (median 21 µg/L, range 2 to 85 g/L) are in line with those measured by a commercial RIA and by others using in-house RIA (mean 15 to 26 µg/L) (Borgström and Ohlsson, 1976, Florholmen et al., 1984b, Geokas et al., 1979), IRMA ("most serum samples do not exceed 70 µg/L") (Lafont et al., 1995), and ELISA (mean 28 µg/L) (Kimland et al., 1989).

We could not make direct comparisons with other assays for trypsinogen-2 because such assays are not generally available. The median trypsinogen-2 concentration in serum measured in our study was 17 µg/L (range 2 to 60 µg/L). Later, a reference range of 18 to 90 µg/L for our assay was determined (Hedström et al., 1994). Similar results have been measured by an ELISA method (mean 21 µg/L) (Kimland et al., 1989). However, Largman et al. (Largman et al., 1978) have determined a normal mean serum level of immunoreactive trypsinogen-2 of 4.5 µg/L. This result was measured by RIA that used TLCK-inactivated trypsin-2 for calibration. The difference in immunoreactivity of trypsinogen-2 and inactivated trypsin-2 was marked at least in our assay and the difference in the standards used could explain the discrepancy.

We found that the ratio of trypsinogen-1 to -2 in sera from healthy subjects and patients with extrapancreatic disease was 1.24. This result is likely to reflect the trypsinogen-1 to -2 ratio of 2 in pancreatic fluid (Figarella et al., 1969, Guy et al., 1978, Rinderknecht et al., 1979). Similar ratio of 1.36 (Kimland et al., 1989) and 1.25 (Petersson et al., 1999) has been reported by others using ELISA.

Acute pancreatitis patients. In acute pancreatitis we found that the serum concentration of trypsinogen-2 is 50-fold higher than in healthy controls, whereas the difference in trypsinogen-1 concentrations was only 15-fold and 10-fold when measured by TR-IFMA and RIA, respectively. Thus, in patients with acute pancreatitis the ratio of serum trypsinogen-1 to -2 was reversed. It was 0.38 as measured by us and 0.77 in another study (Petersson et al., 1999). This finding is compatible with previous reports of the relative concentrations of trypsinogen isoenzymes in pancreatic juice of patients with acute pancreatitis (Borulf et al., 1979, Rinderknecht et al., 1979). As discussed earlier, up-regulation of trypsinogen-2 may be a defensive mechanism, by which trypsin generation is significantly limited in potential pathological conditions (Kukor et al., 2003).

Our finding that the levels of trypsinogen-2 are increased in acute pancreatitis suggested that it could be used as a diagnostic marker for acute pancreatitis. This has been confirmed in other studies both in serum (Hedström et al., 1994, Hedström et al., 1996c, Hedström et al., 2001, Kimland et al., 1989, Kylänpää-Bäck et al., 2002, Rinderknecht, 1996, Sainio et al., 1996) and especially in urine (Appelros et al., 2001, Hedström et al., 1996d, Jang et al., 2007, Kylänpää-Bäck et al., 2000, Sankaralingam et al., 2007). In general, elevated serum levels of trypsinogen-2 are associated with other pancreatic diseases as well, i.e. pancreatic cancer (Borgström and Andren-Sandberg, 1995, Hedström et al., 1996a), chronic pancreatitis (Borgström and Andren-Sandberg, 1995) and pancreas allograft rejection (Douzdjian et al., 1994, Lieberman et al., 1997, Marks et al., 1990, Perkal et al., 1992).

Pancreatectomized patients. The sensitive assays allowed us to measure concentrations of trypsinogen-1 and -2 in serum samples from patients who had undergone pancreatectomy. All samples contained trypsinogen-2 (median 3 µg/L), whereas trypsinogen-1 (2 µg/L) was detected in only one of nine samples. This result showed that the expression of trypsinogen, and especially that of trypsinogen-2, is not restricted to the pancreas. It was earlier shown that human Paneth cells in the intestine express trypsinogen immunoreactivity (Bohe et al., 1984), and the expression of trypsinogen isoenzymes in various normal tissues has now been confirmed by several studies (Cederqvist et al., 2003, Cottrell et al., 2004, Critchley et

al., 2000, Ghosh et al., 2002, Kawano et al., 1997, Koshikawa et al., 1997, Koshikawa et al., 1998, Paju et al., 2000, Stenman et al., 2005, Wiegand et al., 1993).

Immunoreactive trypsinogen-1 and -2 in cancer

Several proteases have been shown to be up-regulated in cancer (Barsky et al., 1983, Diamandis et al., 2003, Hasui et al., 1989, Liotta et al., 1980, Malhotra et al., 2002, de Bruin et al., 1988). We found this to be the case also for TAT. The most interesting finding was that TAT levels, especially those of TAT-2 correlated with the degree of malignancy. We suggested that TAT could promote cellular invasion by participating in the tumor-associated protease cascade involving urokinase plasminogen activator (uPA), uPA receptor (uPAR), plasminogen and latent matrix metalloproteinases (MMPs).

Formation of metastases is the main cause of treatment failure and death for cancer patients. Remodeling of ECM in local invasion is enabled by proteinases like the MMPs and tissue serine proteinases, including tPA, uPA, plasminogen, thrombin, plasmin and trypsin (Mignatti and Rifkin, 1993, Mignatti et al., 1986). Expression of trypsinogen has been found to be associated with aggressiveness of not only ovarian tumors (Hirahara et al., 1995, Hirahara et al., 1998, Paju et al., 2001b, Paju et al., 2004, Stenman et al., 2003), but also esophageal squamous cell carcinoma (Yamamoto et al., 2001), colorectal cancer (Yamamoto et al., 2003), gastric cancer (Ichikawa et al., 2000), experimental gastric cancer in nude mice (Kato et al., 1998), prostate cancer (Bjartell et al., 2005), as well as several human cancer cell lines (Kato et al., 1998, Koshikawa et al., 1992, Miyagi et al., 1995, Miyata et al., 1998, Miyata et al., 1999). The increased expression of TAT-2 can even be measured in serum samples of patients with ovarian cancer (Paju et al., 2004), gastric cancer (Ichikawa et al., 2000), biliary and pancreatic cancer, and cholangiocarcinomas (Hedström et al., 1996a, Hedström et al., 1999, Lempinen et al., 2007).

TAT-2 can directly activate several MMPs (Imai et al., 1995, Koivunen et al., 1989, Moilanen et al., 2003, Nyberg et al., 2002, Paju et al., 2001b, Prikk et al., 2001, Sorsa et al., 1997) and membrane bound latent matriptase (membrane type serine proteinase-1, MT-SP1) (Jin et al., 2005). MT-SP1 degrades extracellular matrix proteins and activates uPa, hepatocyte growth factor (HGF) and PAR-2 (Lee et al., 2000) and its expression is associated with malignancy as well (Lee et al., 2005). TAT-2 has been demonstrated to efficiently degrade many ECM components (Koivunen et al., 1991a, Koshikawa et al., 1992, Moilanen et al., 2003, Stenman et al., 2005). *In vitro* studies have shown that cancer cell mediated degradation of ECM (Koivunen et al., 1991a) and activation of several proMMPs (Moilanen et al., 2003) is inhibited by TATI.

TAT-2 isolated from a colon carcinoma cell line is an efficient activator of PAR-2 in an *in vitro* study (Alm et al., 2000). The PARs are up-regulated in cancer and inflammation (Borgono and Diamandis, 2004) so TATs might also be potential *in vivo* activators of PAR-2. It has thus become widely accepted that TAT plays an important role in cancer progression and metastatic processes such as cellular invasion, degradation of extra-cellular matrix proteins, angiogenesis and tissue remodeling, either alone or in cascade with other proteolytic enzymes.

Immunoreactive TATI in cancer

TATI was earlier shown to be a tumor marker for mucinous ovarian tumors (Halila et al., 1988, Huhtala et al., 1982). This study shows that very high concentrations of TATI, up to 15 000 µg/L, occur in mucinous ovarian cyst fluids, both in benign and malignant ones. We also found that in some serous cyst fluids from borderline and malignant tumors the concentrations of both TAT isoenzymes were remarkably elevated but the levels of TATI were not. We thought this might reflect a disturbance in proteolytic balance, which

may contribute to the invasive properties of malignant cells. Similar results were obtained later, when the molar ratio of trypsinogen to TATI was found to be significantly higher in serous than in mucinous cyst fluids (Paju et al., 2001b).

TAT-2 has been shown to degrade tissue inhibitor of metalloproteinase-1 (TIMP-1) (Sorsa et al., 1997), which might be one factor in disturbing the balance between proteinases and their inhibitors in cancer. The excess of proteinase in relation to its inhibitor was suggested to be related to the poorer prognosis of serous than mucinous ovarian carcinomas at an early stage of the disease by Vergote et al. (Vergote et al., 1993). High TATI expression in gastric cancer tissue seems to correlate with a favourable prognosis for the patient in one study (Wiksten et al., 2005). The finding that trypsinogen is expressed in both malignant and benign bladder epithelium, whereas TATI expression decreases with increasing stage and grade of malignancy, suggests balanced expression of trypsinogen and TATI in normal tissue, but disruption of this balance in tumor progression (Hotakainen et al., 2006). These results are suggestive of a protective role of TATI in tumour invasion, possibly by reducing the proteolytic activity of trypsin and thereby inhibiting tissue destruction and mucosal degradation.

However, in ovarian (Paju et al., 2004, Venesmaa et al., 1994, Venesmaa et al., 1998), bladder (Kelloniemi et al., 2003), prostate (Paju et al., 2007) and renal cell cancers (Paju et al., 2001a) an increased serum level of TATI is a marker of poor prognosis. In a previous report, PSTI/TATI expression correlated, in intestinal type of gastric tumours, with advanced stage tumours as well as nodal involvement (Higashiyama et al., 1990a). It has been suggested that trypsinogen and TATI are expressed simultaneously by many tumors, and an elevation of TATI in serum or urine reflects trypsinogen expression by the tumor, which in most cases is associated with aggressive disease (Paju and Stenman, 2006). Thus, the mechanisms by which trypsins and TATI act in tumor growth and metastasis are not yet understood in detail.

Characterization of pancreatic and extra-pancreatic trypsinogens

Determination of the isoenzyme pattern by ion exchange chromatography revealed isoelectric variants of trypsinogen isoenzymes in serum samples. The less acidic forms corresponded to the main TAT-1 and -2 isoenzymes in mucinous ovarian cyst fluid, and the more acidic isoenzymes corresponded to the main peaks in serum from a patient with pancreatitis. Earlier, pancreatic trypsinogen-1 and -2 had been shown to incorporate radioactive sulfate to tyrosine residue (Scheele et al., 1981). We speculated that lack of sulfation could explain the shorter retention time of TATs in anion exchange chromatography.

Later, based on a crystal structure study and MS analysis of trypsin-1 Gaboriaud et al. (Gaboriaud et al., 1996) localized an 80 Da modifying group at Tyr154 in the substrate binding pocket, in the S'2 subsite. As the mass of HPO_3 is 79.966 Da and that of SO_3 is about 79.957 Da, the PTM was misinterpreted to be phosphate. Szilagyi et al. (Szilagyi et al., 2001) reported that two forms of trypsinogen-1 isoenzymes can be found in human pancreatic juice. Trypsin-1 with a molecular mass 24.184 Da in MS analysis, corresponding to the amino acid sequence of trypsin-1 and an 80 Da mass addition, was obtained from pancreatic juice from one patient. This mass addition was preliminarily suggested to result from sulfation, not phosphorylation. Non-modified trypsin-1 with a molecular mass of 24.104 Da in MS analysis was obtained from another patient's pancreatic juice. The isolation process of this trypsin contained prolonged exposure to acid environment. The authors could not explain whether the non-modified trypsin was result of hydrolytic loss of the modifying group, or was trypsinogen in fact unmodified in that sample (Szilagyi et al., 2001).

Recently, Sahin-Tóth et al. performed alkaline hydrolysis to purified human trypsinogen

isoenzymes (Sahin-Tóth et al., 2006). Thin layer chromatography of the hydrolysates revealed existence of sulfated tyrosine residue in both trypsinogen-1 and -2. The tyrosine sulfate residue was attributed to Tyr154 in trypsinogen-1 by incorporation of $^{35}SO_4$ to trypsinogen-1 but not to Tyr154Phe mutant trypsinogen-1 expressed in human embryonic kidney 239T cells (Sahin-Tóth et al., 2006).

In other words, we observed isoelectric variants of trypsinogen isoenzymes in serum samples by ion exchange chromatography. In the literature, there were contradictory reports of trypsinogen PTM. We therefore isolated pancreatic trypsinogen isoenzymes and TAT-2 from conditioned medium of COLO 205 cell line, and characterized intact trypsinogen isoenzymes, and tryptic and chymotryptic peptides by ESI-MS, Western blot analysis and N-terminal sequencing.

Investigation of Tyr154 modification

Phosphorylation of trypsinogen isoenzymes (Gaboriaud et al., 1996) was excluded by immunoblotting with anti-phosphotyrosine antibody. No specific signal was detected with pancreatic trypsinogen isoenzymes, whereas the antibody efficiently recognized the Tie1 tyrosine kinase, which is known to be phosphorylated on tyrosine (Saharinen et al., 2005). The presence of trypsinogen-1 and -2 isoenzymes, respectively, were verified by probing the same lanes with MAb against the respective trypsinogen isoenzyme.

A clear signal of PHT derivative of Tyr154 was seen in Edman degradation of purified peptide from pancreatic trypsinogen-2. The fact that the PTH derivative of phosphotyrosine is hardly soluble under standard Edman sequencing conditions (Aebersold et al., 1991) indicates the absence of phosphotyrosine in position 154 of trypsinogen-2.

Mass modified trypsin peptides lost their mass addition of 80 Da both in in-source dissociation and CID experiments, which would not occur to phosphorylated peptides. Under the CID conditions in MSMS analysis, the HPO_3 moiety would remain attached to the tyrosine residue allowing site-specific identification of the phosphorylation. Tyrosine phosphorylation in peptides can be identified by detecting the immonium ion of phosphotyrosine (*m/z* 216.04) in positive mode precursor ion scanning on a Q-TOF MS, and the phosphorylation site can be localized in the same experiment by MSMS fragmentation. Furthermore, potential serine or threonine phosphorylation would induce a loss of 98 Da, resulting in the appearance of dehydroalanine or dehydroamino-2-butyric acid in the MSMS fragmentation spectrum under the CID conditions used (Zhou et al., 2001). None of these was observed. In sharp contrast, it is reported (Nemeth-Cawley et al., 2001, Rappsilber et al., 2001) that loss of SO_3 is the first fragmentation event in low-energy collisionally activated dissociation, as the energy required to break the S-O bond is lower than the energy required to fragment the polypeptide backbone. Therefore, the observed loss of 80 Da from both pancreatic trypsinogen-1 and -2 derived peptides in in-source dissociation and CID experiments indicates that they are sulfated and not phosphorylated.

The pancreatic trypsinogen-1 peptide contains only one tyrosine residue, namely Tyr154, while the trypsinogen-2 peptide analyzed contains two tyrosine residues, Tyr154 and Tyr175. Tyr154 is sulfated in trypsinogen-1, and a sulfation consensus sequence surrounds Tyr154 but not Tyr175 in trypsinogen-2. Therefore, it is most probable that the Tyr 154 is also sulfated in trypsinogen-2. Taken together, our results confirm the previous findings indicating that pancreatic trypsinogen-1 and -2 are modified at Tyr154 and that this modification is sulfate, not phosphate (Gaboriaud et al., 1996, Sahin-Tóth et al., 2006, Scheele et al., 1981, Szilagyi et al., 2001).

On contrarily, we could show that TAT-2 from a colon carcinoma cell line is not posttranslationally modified. Instead, in MS analysis the mass of TAT-2 corresponds

to the theoretical mass of trypsinogen-2. This difference in sulfation at Tyr154 could explain the previously reported differences between pancreatic and tumor-associated trypsinogens (Koivunen et al., 1989). Pancreatic trypsinogens have been found to be fully sulfated suggesting extra-ordinary tyrosine sulfation capacity (Sahin-Tóth et al., 2006). TPST-1 and -2 are expressed in all tissues examined (Moore, 2003) but mRNA expression of the TPST-2 isoform is drastically higher in the pancreas than any other tissues examined (Ouyang and Moore, 1998). This might explain the incomplete sulfation of trypsinogens in extra-pancreatic tissues.

Aromatic interactions in proteins

Aromatic interactions, usually described as π-π interactions, are ubiquitous in nature and are involved in many biological processes like in the antigen-binding of immunoglobulins (Padlan, 1990), stability of duplex DNA (Kool, 2001) or stabilizing protein tertiary structures (Mitchell et al., 1994, Singh and Thornton, 1990). The delocalized electrons of the benzene ring are the basis for the these interactions (Kryger et al., 1998, Kryger et al., 1999, Obst et al., 1997).

Contribution of aromatic interactions in binding affinity and ligand selectivity in the S3/S4 pocket of bovine trypsin, human factor Xa and chimeric S3/S4 mutants have been studied (Di Fenza et al., 2007). The aromatic character of this pocket increases from trypsin (only Trp215) to factor Xa (Trp215, Tyr99, Phe174). The results show that the establishment of favourable directional aromatic-aromatic interactions in the S3/S4 pocket with a bound ligand will increasingly contribute to binding affinity and will thus determine selectivity (Di Fenza et al., 2007). Factor Xa is thus more selective with respect to bovine trypsin for ligands which opportunely interact with the fully established aromatic box in the S3/S4 subsite.

The effect of tyrosine sulfation on trypsin

Modification of aromatic tyrosine residue by sulfation provides it with highly polarizable electrons and makes it even more electronegative. Thus, proteins and peptides become more interactive by this PTM (Lyon et al., 2000, Sasaki et al., 1999, Woods et al., 2007). Tyrosine sulfate has been shown to be involved in protein-protein interactions (Costagliola et al., 2002, Stone and Hofsteenge, 1986, Wilkins et al., 1995, Woods et al., 2007) and proteolytic activity (Michnick et al., 1994).

In trypsinogen and trypsin, Tyr154 is located in the S'2 subsite within the primary substrate binding pocket (Gaboriaud et al., 1996, Katona et al., 2002). Thus, it is likely that sulfation of Tyr154 in trypsin contributes to more efficient substrate binding. Indeed, autoactivation of sulfated trypsinogen-1 was shown to be faster than that of the nonsulfated recombinant form (Sahin-Tóth et al., 2006). (Sulfated) pancreatic trypsin-1 and -2 were shown to be more effective activators of pro-uPA than (non-sulfated) TAT-1 and -2, respectively (Koivunen et al., 1989). Furthermore, modified (sulfated) trypsin-1 was shown to be more efficiently inhibited by PSTI than the non-modified form (Sahin-Tóth et al., 2006, Szilagyi et al., 2001).

On contrary to the findings of Sahin-Tóth et al. (Sahin-Tóth et al., 2006) and Szilagyi et al. (Szilagyi et al., 2001), (non-sulfated) TATs were somewhat more efficiently inhibited by TATI and soybean trypsin inhibitor than the pancreatic trypsins, whereas pancreatic trypsin-1 was more efficiently inhibited by limabean trypsin inhibitor than TAT-1 in a study of Koivunen et al. (Koivunen et al., 1989). As described above, there is evidence supporting more efficient substrate binding for the sulfated trypsin forms as compared to the non-sulfated ones. TATI has been found to be heterogenous (Huhtala et al., 1982, Kikuchi et al., 1985) so the PSTI/TATI preparations used in these studies may not necessarily be

comparable. It is also shown that substrate binding is not only determined by the primary substrate binding site, but several distal interactions are also involved (Hedstrom et al., 1992, Hedstrom et al., 1994b). These distal binding interactions and the possible differences in the PSTI/TATI preparations used could explain the discrepancy between these results.

The enzymatic parameters of native (sulfated) pancreatic and non-modified trypsins from pancreatic juice, ovarian cyst fluid and recombinant trypsin expressed in *Escherichia coli* have been determined using *p*-nitroanilide peptide substrates. The kinetic constants of pancreatic and tumor-associated trypsins were similar for one substrate (S-2222), but for two substrates (S-2444 and S-2251) the k_{cat} for pancreatic trypsin-2 was lower and the k_{cat}/K_m higher than that for TAT-2 (Koivunen et al., 1989) indicating for more efficient substrate binding by (sulfated) pancreatic trypsin-2. In other studies using different *p*-nitroanilide peptide substrates, the catalytic activity of native (sulfated) pancreatic trypsin-1, native non-modified pancreatic trypsin-1 and non-modified recombinant trypsin-1, respectively, was found to be practically identical (Sahin-Tóth et al., 2006, Szilagyi et al., 2001). The nonexistent or modest differences reported in the enzymatic parameters between (sulfated) pancreatic trypsins and (non-sulfated) tumor-associated or recombinant trypsins are likely to result from the structure of the *p*-nitroanilide peptide substrates used. The Tyr154 residue in the S'2 subsite interacts with the leaving group side of the scissile bond, not the acyl group side. In the chromogenic substrates used there is no P'2 residue, only the acyl group side (P1 to P4) with an arginine or lysine as the P1 residue. Thus, the influence of S'2 site on substrate binding when using *p*-nitroanilide peptide substrates is unlikely.

Conclusions

The most important result of this study was the development of specific MAbs and TR-IFMAs to trypsinogen-1 and -2. With these we could show that:

1) in acute pancreatitis serum trypsinogen-2 is elevated 50-fold, whereas serum trypsinogen-1 is elevated 15-fold, suggesting that trypsinogen-2 could be a diagnostic marker for acute pancreatitis.

2) TAT-2 is the predominant form in ovarian cyst fluids and its concentrations correlate with malignancy of these tumors. Thus, TAT is likely to be involved in ovarian tumor dissemination and breakage of tissue barriers.

3) serum samples from pancreatectomized patients contain immunoreactive trypsinogen isoenzymes. These results indicate that trypsinogen is not exclusively expressed in the pancreas and certain tumors, but that it may also be produced by normal extrapancreatic tissues.

4) two forms of trypsinogen-1 and -2, respectively, can be found in human sera and ovarian cyst fluids.

Finally, we confirmed by ESI-MS analysis that pancreatic trypsinogen-1 and -2 are sulfated and not phosphorylated at Tyr154, whereas tumor-associated trypsinogen-2 is not. We suggest that this modification may explain the previously observed differences between pancreatic and tumor-associated trypsin.

Concluding remarks

When this study was started PSTI/TATI had been isolated from urine of an ovarian cancer patient (Huhtala et al., 1982). Two tumor-associated trypsinogen (TAT) isoenzymes had been isolated from mucinous ovarian cyst fluid and were suggested to be the target proteinases of TATI in ovarian cancer (Koivunen et al., 1989). It had also been shown by immunohistochemistry that trypsin immunoreactivity occurs in the Paneth cells of the small intestine (Bohe et al., 1986), but the function of this Paneth cell trypsinogen was not known. The human trypsinogen genes were thought to constitute a multigene family of more than ten genes (Emi et al., 1986). It was not known whether the TATs, the Paneth cell trypsinogen, and the pancreatic trypsinogens were encoded by different genes. The production of specific MAbs and development of sensitive time-resolved immunofluorometric assays in the beginning of this study facilitated new approaches to purify and characterize human trypsinogens on one hand, and to study the expression of them in various tissues and diseases on the other hand.

We established provisional reference ranges for trypsinogen-1 and -2 with the newly developed TR-IFMAs. Furthermore, we showed that especially serum trypsinogen-2 levels are strongly elevated in acute pancreatitis. These results are in line with those reported by others (Borgström and Ohlsson, 1976, Florholmen et al., 1984b, Geokas et al., 1979, Hedström et al., 1994, Kimland et al., 1989, Lafont et al., 1995, Petersson et al., 1999). We suggested that serum trypsinogen-2 could be used as a diagnostic marker for acute pancreatitis. Indeed, clinical studies employing the MAbs and TR-IFMAs developed in this study and by others have revealed that trypsinogen-2 in serum and especially in urine is specific and sensitive marker for the diagnosis of acute pancreatitis (Appelros et al., 2001, Hedström et al., 1994, Hedström et al., 1996c, Hedström et al., 1996d, Hedström et al., 2001, Jang et al., 2007, Kimland et al., 1989, Kylänpää-Bäck et al., 2000, Kylänpää-Bäck et al., 2002, Rinderknecht, 1996, Sainio et al., 1996, Sankaralingam et al., 2007). These studies were follwed by the development of a rapid dipstick screening test, which is commercially available (Hedström et al., 1996b). This test is more sensitive and specific than amylase, but due to both tradition and the availability of cheap reagents compatible with automatic clinical chemistry analyzers, serum amylase - despite of its known drawbacks - has remained the most often used marker for acute pancreatitis in hospital laboratories.

Apart from our results, there are no other reports showing that trypsinogen occurs in serum of pancreatectomized patients. However, it is now known that trypsinogen is expressed in several normal tissues other than the pancreas, too. Thus, the levels of trypsinogen measured by us are likely to reflect normal extrapancreatic trypsinogen expression. Trypsinogen-2 was shown to be the main isoenzyme in serum from pancreatectomized patients. It has been shown to be expressed in several extrapancreatic cells (Cederqvist et al., 2003, Ghosh et al., 2002, Koivunen et al., 1989, Koshikawa et al., 1997, Paju et al., 2000, Prikk et al., 2001, Stenman et al., 2005).

Sulfated trypsin(ogen)-1, which is more efficiently autoactivated, more stable and is less sensitive to inhibition than trypsin(ogen)-2, is the main isoenzyme in pancreatic juice (Colomb et al., 1978, Mallory and Travis, 1973, Rinderknecht and Geokas, 1972). This ensures efficient digestion of dietary proteins and activation of other dietary enzymes. It is tempting to speculate that due to the high proteolytic potential of trypsinogen-1 its expression is limited in extrapancreatic tissues, where less proteolytic potential than in digestion is

needed. Trypsinogen-2 (or trypsinogen-3 or -4) would thus remain the main trypsinogen isoenzyme in extrapancreatic tissues.

Our finding that TAT-2 is the predominant trypsinogen form in ovarian cyst fluids and that its concentrations correlate with malignancy led to clinical studies on trypsinogen expression in other malignancies as well. TAT-2 was found to be a new potential diagnostic marker for cholangiocarcinomas (Hedström et al., 1996a, Lempinen et al., 2007) and prognostic marker for ovarian carcinomas (Paju et al., 2004). Up-regulation of TAT-2 has also been found in other cancers (Bjartell et al., 2005, Hotakainen et al., 2006). The methods developed in this study have also been used to clarify the mechanisms underlying tumor growth and metastatic processes (Koivunen et al., 1991a, Lukkonen et al., 2000, Moilanen et al., 2003, Sorsa et al., 1997). The developed MAbs and TR-IFMAs have proved to be excellent tools in the ongoing studies associated with trypsinogen quantitation, purification, and characterization.

The recent development of mass spectrometry, software tools and especially soft ionization techniques has made mass spectrometry a valuable tool in protein chemistry. We were able to determine the chemical difference between pancreatic and tumor-associated trypsinogens by ESI-MS analysis. The absence of sulfation at Tyr154 in tumor-associated trypsinogen is likely to explain the differences between these trypsinogen forms observed earlier. It is possible to produce specific MAbs to sulfotyrosine (Hoffhines et al., 2006, Kehoe et al., 2006). If all extrapancreatic trypsinogens lack sulfate, it would be possible to develop immunometric assays specific for pancreatic trypsinogen-1 and -2. We have become aware of many biological processes other than digestion where pancreatic or extrapancreatic trypsinogens are involved. Specific determination of pancreatic and extrapancreatic trypsinogens, respectively, is therefore of potential clinical utility.

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