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## Human cationic trypsinogen is sulfated on tyrosine-154

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### Abstract

The crystal structure of human pancreatic cationic trypsin showed the chemical modification of Tyr-154, which was originally described as phosphorylation [Gaboriaud et al. (1996) *J Mol Biol* 259, 995–1010]. Here we report that Tyr-154 is sulfated, not phosphorylated. Cationic and anionic trypsinogens were purified from human pancreatic juice and subjected to alkaline hydrolysis. Modified tyrosine amino-acids were separated on a Dowex cation-exchange column and analyzed by thin-layer chromatography. Both human cationic and anionic trypsinogens contained tyrosine-sulfate, but no tyrosine-phosphate, whereas bovine trypsinogen contained neither. Furthermore, incorporation of [<sup>35</sup>S]SO<sub>4</sub> into human cationic trypsinogen transiently expressed by human embryonic kidney 239T cells was demonstrated. Mutation of Tyr-154 to Phe abolished radioactive sulfate incorporation, confirming that Tyr-154 is the site of sulfation in cationic trypsinogen. Sulfated pancreatic cationic trypsinogen exhibited faster autoactivation than a non-sulfated recombinant form, suggesting that tyrosine-sulfation of trypsinogens might enhance intestinal digestive zymogen activation in humans. Finally, sequence alignment revealed that the sulfation motif is only conserved in primate trypsinogens, suggesting that trypsinogen sulfation is absent in other vertebrates.

### Keywords

human cationic trypsin; human anionic trypsin; post-translational modification; tyrosine-sulfation; hereditary pancreatitis

The human pancreas secretes two major trypsinogen isoforms, named cationic and anionic trypsinogens, which share 90 % identity in their primary structure. In addition to their physiological function in digestion, trypsinogens also play an important pathological role in pancreatitis. Mutations that stimulate activation of cationic trypsinogen cause hereditary pancreatitis, whereas a mutation that renders anionic trypsinogen sensitive to degradation was shown to protect against pancreatitis (1,2). In contrast to other known vertebrate trypsinogens, human trypsinogens appear to undergo post-translational modification. In 1996, Gaboriaud et al. (3) solved the crystal structure of human cationic trypsin and described the presence of a phosphate group on Tyr-154 (Tyr-151 in the chymotrypsin numbering system). The authors also carried out mass spectrometry on native trypsin samples and masses consistent with phosphorylation were determined. Corroborating evidence for the presence of a modifying group on human cationic trypsinogen came from Szilágyi et al. in 2001, who confirmed the increased mass of purified native trypsin by mass spectrometry and discussed the possibility that the modifying group might be sulfate instead of phosphate, as both modifications would result in approximately 80 Da extra mass (4). This consideration was in line with an earlier study by Scheele et al., demonstrating that incubation of human pancreatic slices with

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radioactive sodium-sulfate resulted in the incorporation of radioactive sulfate into human trypsinogens (5).

To resolve the controversy about the nature of the modifying group on human trypsinogens, we isolated the tyrosine-sulfate amino-acid from pancreatic trypsinogens and demonstrated radioactive sulfate incorporation into Tyr-154 of cationic trypsinogen transiently expressed in human embryonic kidney (HEK) 293T cells. The experiments presented here conclusively determine that human cationic and anionic trypsinogens are tyrosine-sulfated and not phosphorylated.

## RESULTS

### Electrophoretic analysis of human pancreatic trypsinogens

We purified human cationic and anionic trypsinogens from pancreatic juice and compared their electrophoretic behavior to recombinant trypsinogens expressed in *E. coli*. These recombinant preparations were previously characterized by mass spectrometry and N-terminal sequencing and contain no post-translational modifications (2,4). Analysis on SDS-PAGE under reducing or non-reducing conditions revealed no discernible differences (not shown). On the other hand, when native and recombinant trypsinogens were electrophoresed in the absence of SDS on 13 % "native" polyacrylamide gels, trypsinogens isolated from pancreatic juice migrated faster than their recombinant counterparts (Fig 1). The faster mobility of pancreatic trypsinogens on native gels indicates the presence of extra negative charge(s), which is consistent with a sulfo-tyrosine or phospho-tyrosine amino-acid.

### Demonstration of tyrosine-sulfate in human trypsinogens by alkaline hydrolysis and thin-layer chromatography

To obtain conclusive evidence for the chemical nature of the modifying group, we set out to isolate the modified tyrosine from human trypsinogens and to identify the modifying group by thin-layer chromatography (TLC). In the design of the experimental protocol, we took advantage of the following published observations (6–9). Tyrosine-sulfate and usually tyrosine-phosphate are resistant to alkaline hydrolysis. Due to the presence of the strongly acidic group, tyrosine-sulfate and tyrosine-phosphate do not bind to a Dowex cation exchange column at pH 2.5, whereas under these conditions all other amino-acids remain bound to the resin. Tyrosine-sulfate and tyrosine-phosphate migrate differently on a silica thin-layer chromatography plate in 80 % ethanol solvent. This property allows the clear identification of the modified tyrosine species using appropriate standards.

Human pancreatic trypsinogens were subjected to alkaline hydrolysis, and the modified tyrosines were isolated and analyzed by thin-layer chromatography, as described in *Experimental Procedures*. As a negative control, we used commercial bovine trypsinogen, which is devoid of any phosphate or sulfate modifying groups. Ninhydrin staining of thin-layer silica plates revealed that no tyrosine-sulfate or tyrosine-phosphate amino acids were isolated from bovine trypsinogen hydrolysates (see lanes 1 in Fig 2 left and right panels). In contrast, samples isolated from human cationic trypsinogen or anionic trypsinogen clearly showed a ninhydrin-positive spot (lanes 3 and 5, Fig 2 left panel), which co-migrated with the tyrosine-sulfate standard (lanes 2, 4, 6; Fig 2, left panel). No tyrosine-phosphate was detected in any of the samples analyzed. As shown in Fig 2 (right panel), the tyrosine-phosphate was clearly distinguished from tyrosine-sulfate by its slower migration. Taken together, these experiments unambiguously proved that human trypsinogens contain tyrosine-sulfate, not tyrosine-phosphate.

### Human cationic trypsinogen expressed in HEK 293T cells incorporates [<sup>35</sup>S] from inorganic [<sup>35</sup>S]Na<sub>2</sub>SO<sub>4</sub> into Tyr-154

HEK 293T cells transiently transfected with the cDNA for human cationic trypsinogen secrete trypsinogen into the growth medium. Addition of [<sup>35</sup>S]Na<sub>2</sub>SO<sub>4</sub> to the medium resulted in the incorporation of [<sup>35</sup>S] into the secreted trypsinogen (Fig 3). Importantly, no [<sup>35</sup>S] incorporation was observed into the proteins of conditioned media of mock-transfected HEK 293T cells. Mutation of Tyr-154 to Phe (Y154F) abolished radioactive sulfate incorporation into the major trypsinogen band, confirming that the site of sulfation is Tyr-154 in cationic trypsinogen.

### Tyrosine-sulfation increases autoactivation of human cationic trypsinogen

Catalytic activity of sulfated pancreatic and non-sulfated recombinant cationic trypsins were essentially identical on the chromogenic peptide substrate N-CZB-Gly-Pro-Arg-p-nitroanilide (not shown). On the other hand, a clear difference was observed in autoactivation experiments. Autoactivation of trypsinogens is a self-amplifying proteolytic reaction, in which trypsin activates trypsinogen to trypsin. As shown in Fig 4, sulfated pancreatic cationic trypsinogen autoactivated faster than its recombinant non-sulfated form, particularly at higher calcium concentrations. Interestingly, autoactivation of human anionic trypsinogen seemed unaffected by tyrosine sulfation, as both native and recombinant preparations autoactivated at comparable rates (not shown).

## DISCUSSION

The present study offers conclusive evidence that human trypsinogens undergo post-translational tyrosine-sulfation in the pancreas. Post-translational O-sulfation of tyrosine residues was first described in bovine fibrinopeptide B in 1954 (6), and its common occurrence in proteins of various mammalian cells was demonstrated by Huttner in 1982 (7). In contrast to phosphorylation, tyrosine sulfation is not part of dynamic regulatory mechanisms, as mammalian tissues have no sulfatase activity for the removal of the sulfate moiety (10). Tyrosine-sulfation is catalyzed by membrane-bound tyrosylprotein sulfotransferases (TPST-1 and TPST-2) in the *trans*-Golgi network (11–13). Genetic deletion of TPST-1 in mice causes reduced body weight and increased postimplantation fetal death (14), whereas deletion of TPST-2 results in male infertility (15). The functional role of tyrosine-sulfation has been characterized only in a handful of proteins so far. The most prominent examples include the HIV co-receptor and chemokine receptor CCR5, which requires tyrosine-sulfation in its N-terminal extracellular domain for proper function; the leech-derived thrombin-inhibitor hirudin, which has 10-fold higher affinity in its sulfated form; and P-selectin glycoprotein ligand-1, the sulfation of which is required for high-affinity binding to P-selectin (10 and references therein). In general, tyrosine sulfation is believed to modify protein-protein interactions in various physiological processes (10).

Inspection of the human cationic or anionic trypsinogen sequence in the vicinity of Tyr-154 reveals features characteristic of known tyrosine sulfation sites (10). Thus, acidic residues have been shown to enhance sulfation, particularly on the N-terminal side (–1 position) of the Tyr residue. In addition, turn-inducing residues (Gly, Pro) are often found in the vicinity of sulfated Tyr residues, but basic residues (Arg, Lys) are generally excluded. In human trypsinogens, the Gly-Ala-Asp-Tyr-Pro-Asp-Glu sequence contains 3 acidic residues, one N-terminal (–1) and two C-terminal (+2 and +3) of the sulfated Tyr-154. Furthermore, the presence of two turn-inducing residues (Gly-151 and Pro-155) and the absence of any basic residues (Arg or Lys) are also readily apparent. Thus, Tyr-154 seems to conform to the general criteria of tyrosine sulfation sites. This sequence is fully conserved in all 3 human trypsinogens. Genomic sequences of the chimpanzee and the rhesus monkey predict that all potentially expressed trypsinogens contain the human sulfation motif (Table 1). A probably innocuous Gly→Thr

change is noted in the chimpanzee mesotrypsinogen sequence at position -3. In stark contrast to the primate trypsinogens, the sulfation motif is absent in all other mammalian, avian, amphibian or fish trypsinogens. Although Tyr-154 *per se* is conserved in the majority of the sequences examined, the potentially critical Asp-153 is uniformly absent. Furthermore, one or both of the two C-terminal acidic residues (Asp-156 and Glu-157 in human) are also missing in most trypsinogens. These structural differences, particularly the absence of the N-terminal (-1) acidic residue, suggest that all known animal trypsinogens are unmodified at Tyr-154 and tyrosine-sulfation of pancreatic trypsinogens is primate-specific.

It is interesting to note that in our experiments human pancreatic trypsinogens appeared fully sulfated, as judged by native gel electrophoresis or by MonoQ anion-exchange chromatography. Given the fact that trypsinogens are among the most abundant secretory proteins, the human pancreas seems to exhibit extraordinary tyrosine-sulfating capacity. In this respect, Ouyang and Moore (1998) reported that mRNA expression of the TPST-2 isoform was drastically higher in the pancreas than any other tissues examined (13). This observation also raises the possibility that trypsinogens expressed in extra-pancreatic tissues may be incompletely sulfated.

The functional significance of tyrosine sulfation in human trypsinogens remains obscure at this time. Tyr-154 forms part of the S2' subsite on trypsin and thus might modify interactions with various substrates and inhibitors. In this respect, Szilágyi et al. (2001) measured binding of pancreatic secretory trypsin inhibitor to native (sulfated) and recombinant (non-sulfated) trypsin and found only a 2-fold difference in the equilibrium dissociation constants (4). In our preliminary experiments, native cationic trypsinogens exhibited faster autoactivation than recombinant cationic trypsinogen, particularly in the presence of higher calcium concentrations. In contrast, autoactivation of anionic trypsinogen was unaffected. Because cationic trypsinogen is the predominant trypsinogen isoform secreted, these observations suggest that tyrosine-sulfation might be important for efficient zymogen activation in the human digestive tract.

## EXPERIMENTAL PROCEDURES

### Materials

De-identified samples of pure human pancreatic juice were received in lyophilized form, as a kind gift from Dr. Niels Teich (University of Leipzig, Germany). The juice was collected from patients who underwent pancreas transplantation with bladder drainage of exocrine pancreatic secretions. To prevent zymogen activation, Trasylol (aprotinin) was added at the time of collection. See ref (16) for details. Carrier-free [<sup>35</sup>S]Na<sub>2</sub>SO<sub>4</sub> (1 mCi; 10 mCi/mL in water) was purchased from American Radiolabeled Chemicals, Inc (St. Louis, Missouri). Tyrosine-sulfated cholecystokinin fragment 26–33 (CCK-8) was obtained from Peptides International (Louisville, Kentucky). The CCK-8 peptide (0.52 mg) was hydrolyzed and the tyrosine-sulfate was isolated as described below. Dowex 50WX2-400 cation-exchange resin and tyrosine-phosphate was from Sigma (St. Louis, Missouri). Tyrosine-phosphate was dissolved in water at 1 mM concentration. F-12 nutrient mixture (Ham) containing 1 mM L-glutamine was purchased from Invitrogen (Carlsbad, California). Whatman flexible plates for TLC (AL SIL G; 20 × 20 cm; aluminum backing; silica gel coating, 250 μM layer) were obtained from Fisher Scientific. Ninhydrin, ozone friendly ready-to-use spray for TLC (in 2-propanol as solvent) was from Acros Organics (Geel, Belgium).

### Purification of native trypsinogens from pancreatic juice

Lyophilized pancreatic juice powder (50–100 mg) was solubilized with 1 mL 10 mM HCl, and insoluble material was removed by centrifugation. The supernatant was diluted with 3 mL

water and loaded onto a MonoQ anion-exchange column (Pharmacia) equilibrated with 20 mM Tris-HCl (pH 8.0). Proteins were eluted with a 0-0.5 M NaCl gradient at 1 mL/min flow rate and 1 mL fractions were collected. The two trypsinogen peaks were collected and further purified with ecotin-affinity chromatography. See ref (17) for more detail.

### Expression of recombinant trypsinogens in *Escherichia coli*

Expression plasmids and strains, growth conditions; isolation of inclusion bodies, in vitro refolding and ecotin-affinity chromatography were described previously (18–20).

### Isolation and identification of tyrosine-sulfate from native trypsinogens by thin-layer chromatography

Approximately 40 nmol (1 mg) of purified trypsinogens were dialyzed against 1 mM HCl and lyophilized. The protein was re-suspended in 480  $\mu$ L of water and 20  $\mu$ L 5 N NaOH was added to a final concentration of 0.2 N. Trypsinogens were then hydrolyzed at 100 °C for 16–18 hours in glass vials. The sample was cooled to room temperature and 500  $\mu$ L 1 M HCOOH was added to acidify the solution. The 1 mL samples were loaded onto Dowex mini-columns (0.8 mL resin equilibrated with 0.1 M HCOOH), and the columns were washed with 0.1 M HCOOH. Fractions (1 mL) were collected, lyophilized, re-suspended in 100  $\mu$ L water and dried again. Finally, the dried material was re-suspended in 10  $\mu$ L of water and 0.5  $\mu$ L samples were analyzed by thin-layer chromatography on silica plates in 80 % ethanol as solvent. Amino-acids were visualized by spraying with ninhydrin and heating with a blow dryer. The developed plates were then scanned for documentation.

### Transfection of HEK 293T cells and metabolic labeling with [<sup>35</sup>S]Na<sub>2</sub>SO<sub>4</sub>

The pcDNA3.1 expression plasmid containing the gene for human cationic trypsinogen was described previously (17). Mutation Y154F was introduced by oligonucleotide-directed site-specific mutagenesis using the overlap extension PCR method. HEK 293T cells were grown in Dulbecco's modified Eagle's medium (DMEM, Invitrogen) with 10 % fetal bovine serum (FBS), and 4 mM L-glutamine to 95 % confluence in 60 mm Petri-dishes. Transfections were carried out in Opti-MEM I reduced-serum medium with 2 mM L-glutamine (Invitrogen) using 8  $\mu$ g plasmid DNA and 20  $\mu$ L Lipofectamine 2000™ reagent (Invitrogen). After 5 hours, the medium was supplemented with DMEM and FBS to a 10 % final concentration. After 24 hours, cells were washed with sulfate-free F-12 medium (Ham); covered with 2.5 mL of the same medium and supplemented with 10  $\mu$ L [<sup>35</sup>S]Na<sub>2</sub>SO<sub>4</sub> (10 mCi/mL). Conditioned medium was harvested after 48 h incubation.

## ACKNOWLEDGMENTS

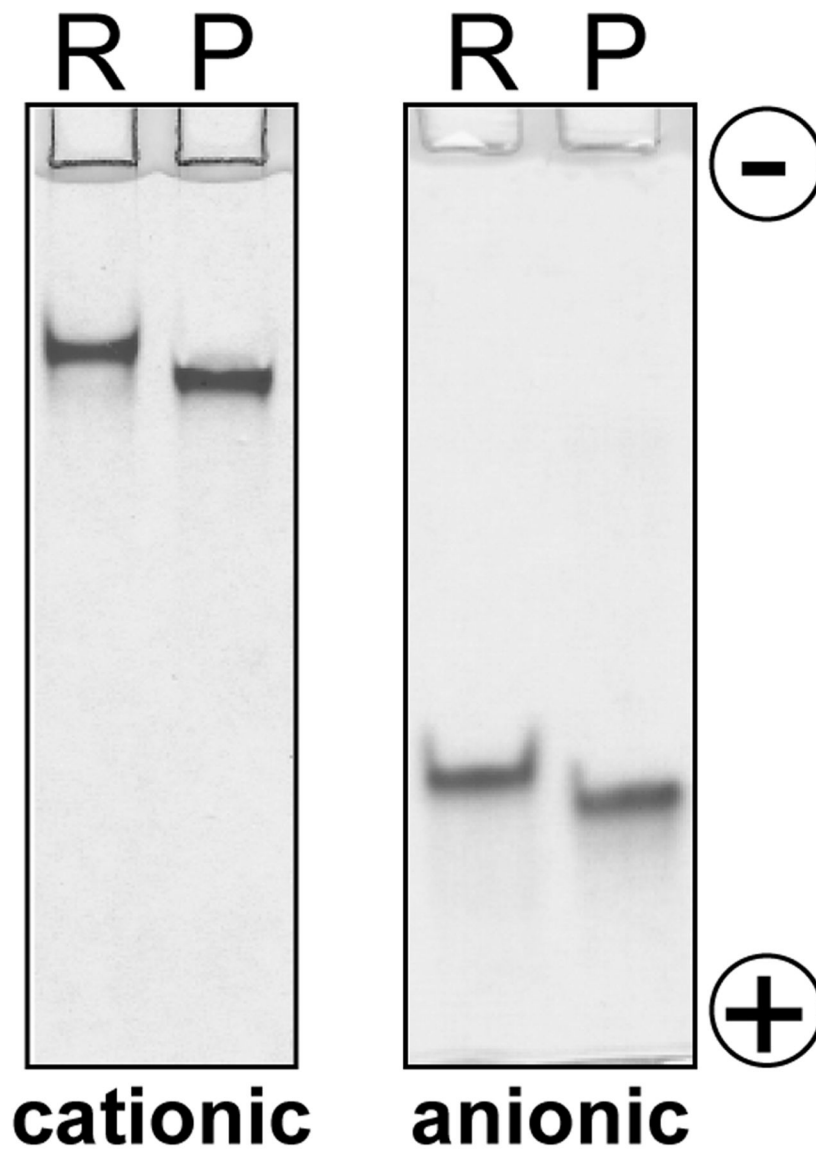
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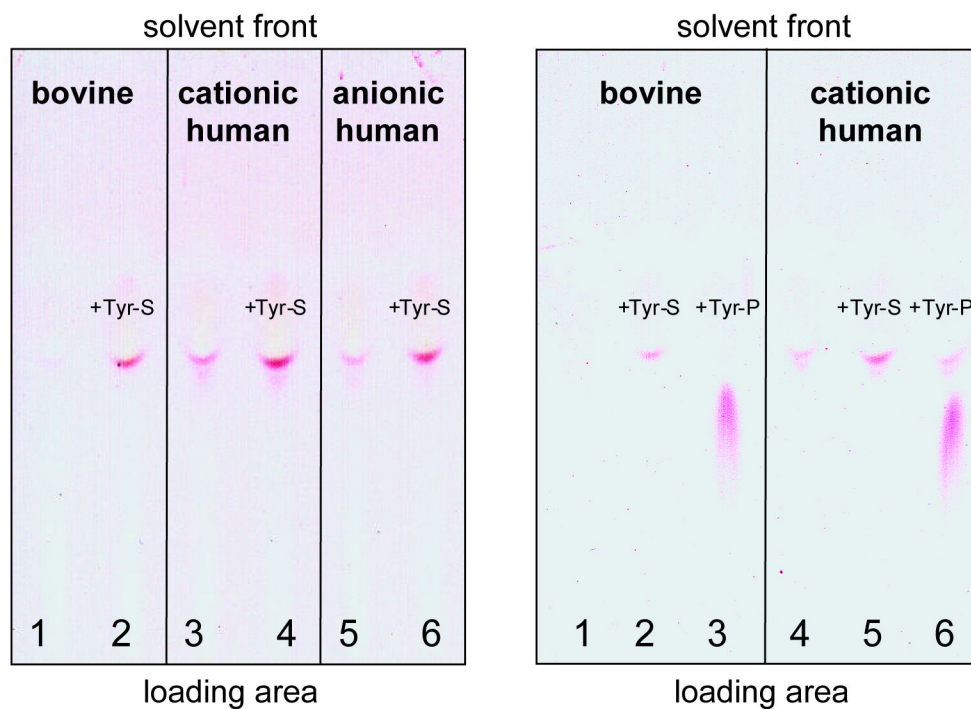
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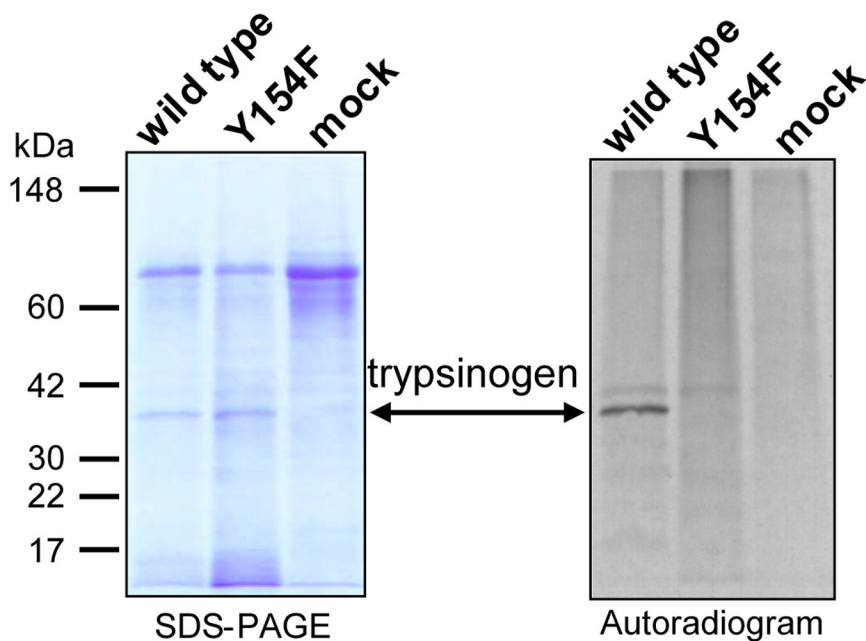
**Figure 1.** Native gel electrophoresis of pancreatic (**P**) and recombinant (**R**) human cationic and anionic trypsinogens. Pancreatic human trypsinogens were purified from pancreatic juice. Recombinant human trypsinogens were expressed in *E. coli*. Aliquots (~10  $\mu$ g) of purified trypsinogens were lyophilized and re-suspended in native sample buffer devoid of SDS or reducing agents. Samples were resolved on 13 % polyacrylamide gels in Tris-glycine buffer (pH 8.5) and the gels were stained with Coomassie blue.



**Figure 2.**

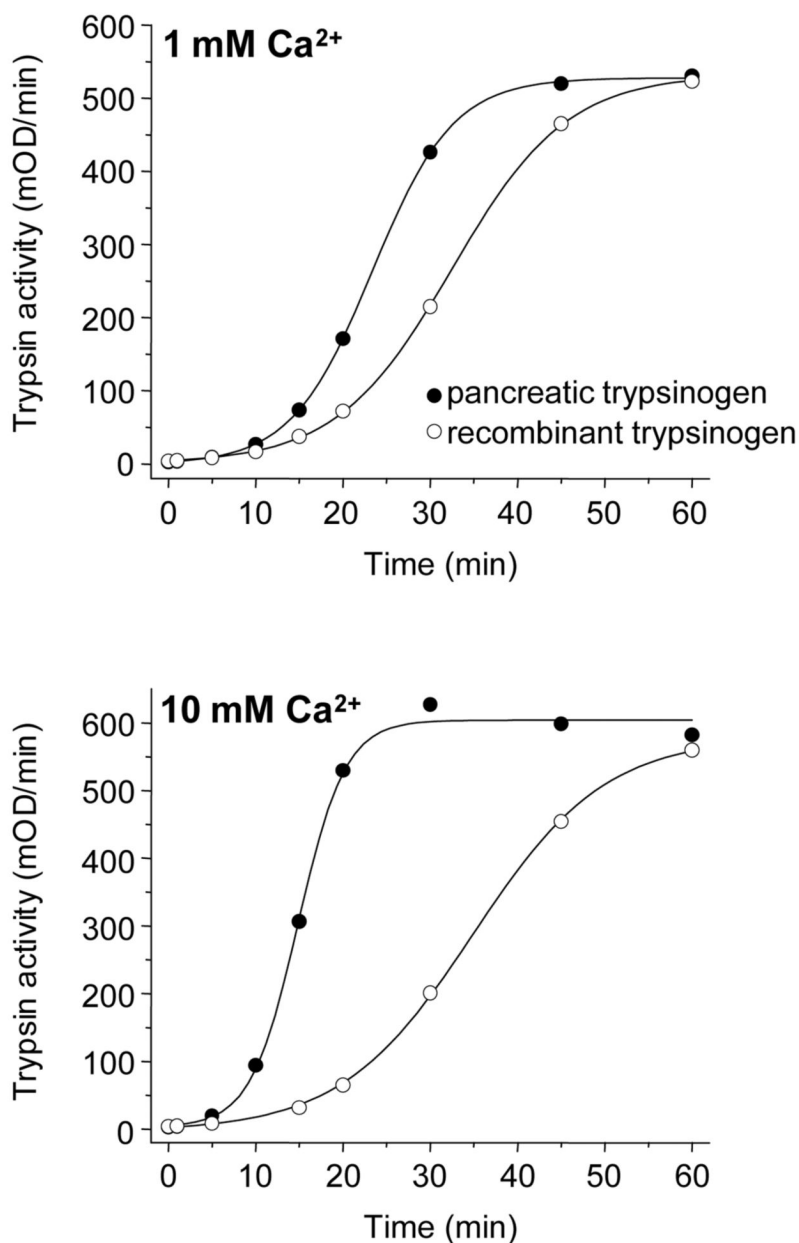
Thin-layer chromatography of tyrosine-sulfate isolated from human trypsinogens after alkaline hydrolysis. The silica plate was developed using 80 % ethanol as solvent. The plate was then air-dried, sprayed with ninhydrin solution and heated with a hair-dryer. See text for experimental details. Tyrosine sulfate (Tyr-S) and (Tyr-P) standards were co-loaded with the trypsinogen-derived samples where indicated.





**Figure 3.**

Incorporation of [ $^{35}\text{S}$ ]SO $_4$  into Tyr-154 of human cationic trypsinogen expressed in human embryonic kidney (HEK) 293T cells. Transiently transfected HEK 293T cells expressing wild-type or Y154F mutant trypsinogens were incubated with [ $^{35}\text{S}$ ]Na $_2$ SO $_4$  in F-12 medium, as described in *Experimental Procedures*. Conditioned media were harvested 48 h after transfection and 200  $\mu\text{L}$  (wild-type) or 400  $\mu\text{L}$  (Y154F and mock) medium was precipitated with 10 % trichloroacetic acid (final concentration). The precipitate was recovered by centrifugation, resuspended in Laemmli sample buffer with 100 mM dithiothreitol, heat-denatured and on run on a 13 % gel. The gel was stained with Coomassie blue, dried between two cellophanes, and exposed to film for 48 h. The markers used were MultiMark pre-stained standards from Invitrogen. The faint bands above the main trypsinogen band on the autoradiogram are probably aberrantly glycosylated trypsinogens, which become visible due to [ $^{35}\text{S}$ ]SO $_4$  incorporation into their sugar moiety.



**Figure 4.**

Autoactivation of pancreatic (solid circles) and recombinant (empty circles) cationic trypsinogens. Trypsin-mediated trypsinogen activation was measured at 37 °C, using 2  $\mu$ M trypsinogen and 10 nM trypsin initial concentrations in 0.1 M Tris-HCl (pH 8.0), 2 mg/mL bovine serum albumin and the indicated CaCl<sub>2</sub> concentrations. At given times, 2  $\mu$ L aliquots were removed from the activation reactions and trypsin activity was measured with the synthetic chromogenic substrate, *N*-CBZ-Gly-Pro-Arg-*p*-nitroanilide at 0.14 mM final concentration. One-minute time-courses of *p*-nitroaniline release were followed at 405 nm in 0.1 M Tris-HCl (pH 8.0), 1 mM CaCl<sub>2</sub>, at room temperature using a Spectramax Plus 384 microplate reader (Molecular Devices). The slope of the linear portion of the absorbance

increase was expressed in mOD/min units. The maximal trypsin activity was 600 mOD/min. The calculated rates of autoactivation of recombinant and pancreatic trypsinogens were 1.5 nM/min and 2.1 nM/min in 1 mM CaCl<sub>2</sub> and 1.5 nM/min and 3.6 nM/min in 10 mM CaCl<sub>2</sub>, respectively.

**Table 1**

Alignment of the human trypsinogen sulfation motif (from Gly-151 to Glu-157) with the homologous sequences of vertebrate trypsinogens.

Human cationic	GADYPDE
Human anionic	GADYPDE
Human mesotrypsinogen	GADYPDE
Chimpanzee anionic	GADYPDE
Chimpanzee C	GADYPDE
Chimpanzee mesotrypsinogen	TADYPDE
Rhesus monkey (all isoforms)	GADYPDE
Bovine cationic	GTSYPDV
Bovine anionic	GVNYPDL
Pig cationic	GSSYPSL
Dog cationic	GQNYPDV
Dog anionic	GTNYPEL
Rat 1	GVNNPDL
Rat 2	GVNEPDL
Rat 3	GTNYPSL
Rat 4	GGKYPAL
Rat 5	GFESPSV
Mouse 7	GTNYPSL
Mouse 4, 5	GGKYPAL
Mouse 8, 9, 20	GVNNPDL
Guinea pig	GVKNPDL
Ostrich	GSSFPEI
Chicken I-P38	GSLYPDV
Chicken II-P29	GYNYPEL
Chicken I-P1	GSLYPDV
Xenopus laevis 1	GSNYPDL
Xenopus laevis 2	GTNYPDL
Xenopus tropicalis 1	IGEFPDR
Xenopus tropicalis 10	GTNYPDL
Atlantic salmon 1	STADSNK
Atlantic salmon 2	STADKNK
Atlantic salmon 3	SSNYPDT
Chum salmon anionic	STADKNK
Zebrafish	GSNYPSR
Atlantic cod 1	SVADGDK
Atlantic cod Y	EVNLPTR
Pufferfish 1	DVFNPFN
Pufferfish 2	ADRNKLQ
Japanese flounder 1	STANRDM
Japanese flounder 2	SSTDSSR
Japanese flounder 3	QVFNPFN