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RESEARCH ARTICLE | *Pancreatic Physiology/Pathophysiology*

Detection of human elastase isoforms by the ScheBo Pancreatic Elastase 1 Test

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Tóth AZ, Szabó A, Hegyi E, Hegyi P, Sahin-Tóth M. Detection of human elastase isoforms by the ScheBo Pancreatic Elastase 1 Test. *Am J Physiol Gastrointest Liver Physiol* 312: G606–G614, 2017. First published March 30, 2017; doi:10.1152/ajpgi.00060.2017.—Determination of fecal pancreatic elastase content by ELISA is a reliable, noninvasive clinical test for assessing exocrine pancreatic function. Despite the widespread use of commercial tests, their exact molecular targets remain poorly characterized. This study was undertaken to clarify which human pancreatic elastase isoforms are detected by the ScheBo Pancreatic Elastase 1 Stool Test and whether naturally occurring genetic variants influence the performance of this test. Using recombinantly expressed and purified human pancreatic proteinases, we found that the test specifically measured chymotrypsin-like elastases (CELA) 3A and 3B (CELA3A and CELA3B), while CELA2A was not detected. Inactive proelastases, active elastases, and autolyzed forms were detected with identical efficiency. CELA3B elicited approximately four times higher ELISA signal than CELA3A, and we identified Glu^{154} in CELA3B as the critical determinant of detection. Common genetic variants of CELA3A and CELA3B had no effect on test performance, with the exception of the CELA3B variant W79R, which increased detection by 1.4-fold. Finally, none of the human trypsin and chymotrypsin isoforms were detected. We conclude that the ScheBo Pancreatic Elastase 1 Stool Test is specific for human CELA3A and CELA3B, with most of the ELISA signal attributable to CELA3B.

NEW & NOTEWORTHY The ScheBo Pancreatic Elastase 1 Stool Test is widely used to assess pancreatic exocrine function, yet its molecular targets have been poorly defined. We demonstrate that, among the human pancreatic proteinases, the test measures the elastase isoform CELA3B and, to a lesser extent, CELA3A. Genetic variants of the human CELA3 isoforms have no significant effect on test performance.

pancreas; digestive proteinase; elastase; chronic pancreatitis; pancreatic insufficiency

DISEASES OF THE PANCREAS that result in loss of functional acinar cells can compromise digestive enzyme production and eventually lead to maldigestion. Clinical laboratory tests that quantify decreased digestive enzyme output can aid in the diagnosis of pancreatic insufficiency. The most widely used tests measure levels of a pancreatic elastase enzyme in the stool (1, 7, 8, 12, 13, 15, 18-20, 23, 30, 32, 44). The chymotrypsin-like elastases (CELAs) are digestive serine proteinases secreted by the pancreas. CELA1 was first described in 1949 by the Hungarian scientists Baló and Banga as an enzyme in the pig pancreas capable of hydrolyzing insoluble elastin (3, 4). Because of its cationic character, CELA1 can absorb to the surface of the negatively charged elastin fibers and cleave multiple Ala-Ala and Ala-Gly peptide bonds (9, 10, 43). Despite its name, CELA1 is not a specific elastin-degrading enzyme, and it readily digests a variety of dietary protein substrates. The primary specificity pocket of CELA1 accommodates small (Ala and Ser) and aliphatic (Ile, Leu, Met, and Val) amino acid side chains at the so-called P1 position of its substrates [Schechter-Berger nomenclature (29) of proteinase-substrate interactions, where P1-P1′ corresponds to the scissile peptide bond] (Ref. 5 and references therein). Curiously, while the human *CELA1* gene appears to be potentially functional, it is not expressed in the pancreas because of evolutionary mutations in its promoter and enhancer regions (26, 41). A second pancreatic elastase (CELA2) was identified on the basis of its ability to solubilize elastin (Ref. 17; for a complete list of references see Ref. 5). Unlike CELA1, this elastase exhibits chymotrypsin-like P1 specificity and prefers to cleave after aromatic (Tyr and Phe) and aliphatic (Leu and Met) P1 amino acids (6, 17, 34). In humans, evolutionary duplication of *CELA2* gave rise to the *CELA2A* and *CELA2B* genes. Even though both genes are expressed at the mRNA level (14), only the CELA2A enzyme is functional, as CELA2B seems to have accumulated inactivating evolutionary mutations (35). The CELA2A content of pancreatic juice corresponds to ~10% of total protein (24).

Arguably, CELA3 has the most interesting history and characteristics among the human elastases. This elastase gene is also duplicated in humans, and the two closely related isoforms were designated CELA3A and CELA3B (31, 42). Both are expressed in the pancreas at comparable mRNA and protein levels (31, 42). Substrate specificity of human CELA3A and CELA3B appears to be similar to that of porcine CELA1, broadly directed toward aliphatic P1 side chains (Ref. 5 and references therein). CELA3B was first described in 1975 as protease E, an anionic pancreatic proteinase devoid of elastolytic activity (21). A subsequent study in 1976 isolated human CELA3B and CELA2A and designated these enzymes elastase 1 and elastase 2, respectively (17). For reasons that remain unclear, the authors found that CELA3B was capable of solubilizing elastin, an erroneous observation, which, at the time, justified the elastase 1 name. Finally, in a number of

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studies starting in 1982, Sziegoleit and co-workers characterized a so-called cholesterol-binding protein with proteolytic activity, which eventually was found to correspond to CELA3B (36, 37, 39). These authors also determined that the CELA3B content of human pancreatic juice accounts for 4–6% of total protein (36). Thus the combined levels of CELA3A and CELA3B are similar to that of CELA2A. Spurred by the observation that CELA3B suffers no proteolytic degradation during intestinal transit and appears in the stool in high concentrations (38), ELISA tests have been developed for the detection of stool elastase, and their clinical utility in the diagnosis of pancreatic insufficiency has been demonstrated (7, 8, 12, 15, 18-20, 23, 30, 32). One of the most widely used assays is the ScheBo Pancreatic Elastase 1 Stool Test (ScheBo Biotech, Giessen, Germany), which utilizes two monoclonal antibodies raised against CELA3B to measure enzyme levels. However, it remains unclear whether the test also detects other elastases, CELA3A in particular, and the extent to which the homologous pancreatic trypsins and chymotrypsins might interfere with the assay. More importantly, the potential confounding effect of natural CELA3 variants on test performance has not been evaluated. In the present study we set out to fill these knowledge gaps, and using well-defined recombinant pancreatic proteinases, we characterized the detection specificity of the ScheBo ELISA test.

MATERIALS AND METHODS

Materials. The ScheBo Pancreatic Elastase 1 Stool Test was purchased from the manufacturer. For some of the experiments, we used the ScheBo Pancreatic Elastase 1 Serum Test, which contains essentially the same ELISA components.

Nomenclature. Coding DNA numbering starts with the first nucleotide of the translation initiator codon, which was designated c.1. Amino acid residues were numbered starting with the initiator methionine of the primary translation product. Note that the *CELA3A* and *CELA3B* genomic reference sequences (chromosome 1 primary assembly, NC_000001.11) contain the minor allelic variants G241A and W79R, respectively. In the present study we used the common alleles at these positions as the reference and designated G241A and W79R the minor variants.

Plasmid construction and mutagenesis. Expression plasmids for human elastases CELA2A, CELA3A, and CELA3B and chymotrypsins CTRB1, CTRB2, CTRC, and CTRL1 constructed in the pcDNA3.1(−) vector (Ref. 33 and references therein) and for human trypsins PRSS1, PRSS2, and PRSS3 in the pTrapT7 vector are described elsewhere (16, 27, 28, 40). The plasmids contain the coding DNA for the proenzyme (zymogen) form of the indicated pancreatic proteinases. Mutations in *CELA3A* and *CELA3B* were introduced by overlap extension PCR mutagenesis. The coding DNA for autolyzed forms of CELA3B carrying nine His residues at their COOH terminus was created by gene synthesis and cloned into the pcDNA3.1(−) plasmid using *Eco*RI and *Bam*HI restriction sites. In the CELA3B-9del construct, the NH_2-terminal nine amino acids from Tyr^{18} to Ser^{26} were deleted. In the CELA3B-13del construct, the NH_2-terminal 13 amino acids from Tyr^{18} to Val^{30} were removed.

Cell culture and transfection. For small-scale expression studies, HEK 293T cells were grown in six-well tissue culture plates (1.5×10^6 cells per well) in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, 4 mM glutamine, and 1% penicillin-streptomycin at 37°C. Transfections were performed with 4 μg of expression plasmid with 10 μl of Lipofectamine 2000 in 2 ml of DMEM. After overnight incubation, cells were rinsed and covered with 2 ml of Opti-MEM reduced-serum medium. The conditioned media were harvested after 48 h.

Purification of pancreatic proteinases. Human proelastases and chymotrypsinogens were expressed in transiently transfected HEK 293T cells and purified from the conditioned medium through their COOH-terminal His tags by nickel-affinity chromatography, as reported elsewhere (33). Human trypsinogens were expressed in *Escherichia coli* as insoluble inclusion bodies. Refolding and purification on immobilized ecotin were performed according to our published protocol (16, 27, 28, 40). Concentrations of proenzyme solutions were determined on the basis of their UV absorbance at 280 nm with use of the following extinction coefficients, calculated with the web-based ProtParam tool (in $M^{-1} \cdot cm^{-1}$): 73,505 for CELA2A, 76,025 for CELA3A, 74,535 for CELA3B, 69,035 for CELA3B mutant W79R, 47,605 for CTRB1 and CTRB2, 64,565 for CTRC, 37,525 for PRSS1, 38,890 for PRSS2, and 41,535 for PRSS3. Proteinase solutions were diluted to 1 nM working stocks in assay buffer [0.1 M Tris·HCl (pH 8.0), 1 mM $CaCl_2$, and 0.05% Tween 20].

Expression and purification of autolyzed CELA3B forms. The CELA3B-9del and CELA3B-13del constructs were produced in HEK 293T cells and purified by nickel-affinity chromatography as described for the wild-type proenzyme (33). NH_2-terminal sequencing of the purified proteins revealed that ~90% of the CELA3B-9del preparation contained the expected NH_2 terminus of Ser^{27} and minor contaminants with NH_2-terminal amino acids of Asn^{31} (5%) and Glu^{33} (5%). The purified CELA3B-13del contained a ~50:50 mixture of two forms, one with the expected NH_2 terminus of Asn^{31} and another with an NH_2-terminal Glu^{33}, indicating that the signal peptidase processed this construct at two sites. This preparation was suitable for the purpose of our experiments, and further purification was not attempted.

Enzyme activity measurements. Enzyme activity of human CELA3A and CELA3B in the conditioned medium of transfected cells was determined using the Suc-Ala-Ala-Pro-Ala-*p*-nitroanilide substrate (5). To activate proelastases, aliquots of conditioned media (100 μl) were supplemented with 10 μl of 1 M Tris·HCl (pH 8.0) and 1 μl of 0.1 M $CaCl_2$ and incubated with 100 nM human cationic trypsin at 37°C for 30 min (final concentrations). Activated elastases (20 μl) were then mixed with 175 μl of assay buffer [0.1 M Tris·HCl (pH 8.0), 1 mM $CaCl_2$, and 0.05% Tween 20], and elastase activity was measured by addition of 5 μl of 6 mM substrate. The increase in absorbance at 405 nm was followed for 5 min in a microplate reader at 22°C. Rates of substrate cleavage were calculated from the linear portion of the curves and expressed in milli-optical density units per minute.

ELISAs. Detection of human pancreatic proteinases by the ScheBo test was performed as follows according to the manufacturer's instructions with the ready-to-use reagents supplied. The 5× wash buffer [phosphate-buffered saline (pH 7.2) with unspecified detergent] stock was diluted with water before use. Aliquots (50 μl) of purified proteinases or conditioned media diluted in assay buffer [0.1 M Tris·HCl (pH 8.0), 1 mM $CaCl_2$, and 0.05% Tween 20] were added to the ELISA strips containing an immobilized anti-elastase antibody (Fig. 1). As blank, 50 μl of assay buffer were used. After 30 min of incubation at 22°C, the enzyme solutions were removed from the wells. The wells were rinsed three times for 2 min each with 250 μl of wash buffer. An aliquot (50 μl) of a biotinylated anti-elastase antibody complexed with peroxidase-conjugated streptavidin was added to the wells, and the sample was incubated for 30 min in darkness at 22°C. The antibody solution was discarded, and the wells were rinsed three times for 2 min each with 250 μl of wash buffer. For color development, 100 μl of 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)-peroxidase substrate solution were added, and the sample was incubated in darkness for 20 min at 22°C. The reaction was terminated by addition of 100 μl of stop solution and incubation for 10 min. The dark-green ELISA signal (absorbance) was measured in a plate reader at 405 nm. All assays were performed in duplicates.

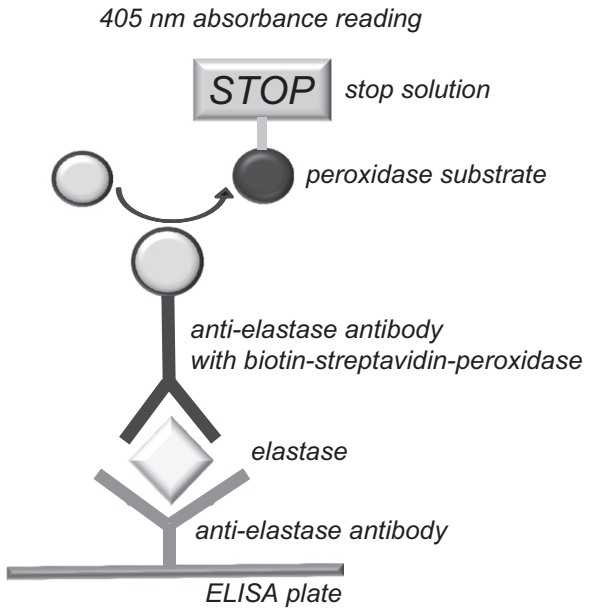


Fig. 1. Schematic diagram of elastase detection by the sandwich ELISA method of the ScheBo Pancreatic Elastase 1 Stool Test.

Data points represent absorbance readings corrected for the average of two blank values.

RESULTS

The ScheBo Pancreatic Elastase 1 Stool Test detects elastase 3 isoforms CELA3A and CELA3B. To determine the specificity of the ScheBo test, we recombinantly expressed and purified human pancreatic serine proteinases and performed ELISAs according to the manufacturer's instructions. CELA3A and CELA3B were tested at 100 pM final concentration; all other proteinases were tested at the 10-fold-higher 1 nM concentration (Fig. 2). We obtained strong signals for CELA3A and CELA3B, whereas none of the other proteinases was detected to a significant extent.

Proelastases, active elastases, and autolyzed elastase forms are measured with equal efficiency. To characterize the detection of CELA3A and CELA3B by the ScheBo test in a more quantitative manner, we performed the ELISA using elastases over a concentration range of 20–200 pM. As shown in Fig. 3*A*, the ELISA signal strength for CELA3B was, on average, 4.3-fold (range 3.4- to 5.1-fold) greater than that for CELA3A over the concentration range tested. Identical signals were obtained when proelastases were compared with active elastases, indicating that the test measures the zymogen and active forms with equal efficiency (Fig. 3*A*). Autolyzed forms of CELA3B missing either 9 amino acids (9del) or 13 amino acids (13del) from the NH_2 terminus also produced ELISA signals identical to that of the intact CELA3B proelastase (Fig. 3*B*). Since these results indicate that the ScheBo test does not discriminate between proelastase, active elastase, and autolyzed elastase, in all subsequent experiments we used the proelastase forms of CELA3A and CELA3B. In a control experiment we also ruled out the unlikely confounding effect of the His tag on elastase expression and detection by the ScheBo test. Although data are not shown, tagged and untagged forms of CELA3A and CELA3B were secreted at comparable levels into the conditioned medium of transfected cells and gave identical ELISA signals.

Effect of CELA3A and CELA3B genetic variants on performance of the ScheBo test. To identify genetic variants of *CELA3A* and *CELA3B* in the population, we interrogated the Exome Variant Server database of the National Heart, Lung, and Blood Institute Exome Sequencing Project. When considering missense variants with an allele frequency >1%, we found one *CELA3A* variant (G241A) and five *CELA3B* variants (W79R, Q134L, I209V, R210H, and A241G) (see Table 1 in Ref. 33 for further details). The occurrence of *CELA3A* variant G241A and *CELA3B* variant A241G was also confirmed by our recent genetic study in a Hungarian population (25). To evaluate whether common genetic variants of *CELA3A* and *CELA3B* might alter performance of the ScheBo test, we purified these six variants and tested their detection in the ELISA. With the exception of the CELA3B W79R variant, none of the variants had an appreciable effect on signal development (Fig. 4). The strength of the signal for variant W79R was, on average, 1.4-fold (range 1.2- to 1.5-fold) greater than that for wild-type CELA3B over the concentration range tested. However, if we consider that in most carriers the variant is heterozygous, this difference should have no meaningful impact on the clinical interpretation of the ScheBo test results.

Glu^{154} in CELA3B is a critical determinant of recognition by the ScheBo test. CELA3A and CELA3B share 92% identity at the amino acid level, yet the strength of the ELISA signal is nearly fourfold greater for CELA3B than CELA3A. To identify the reason for this difference, we aligned the two isoforms (Fig. 5*A*) and then individually mutated all divergent amino

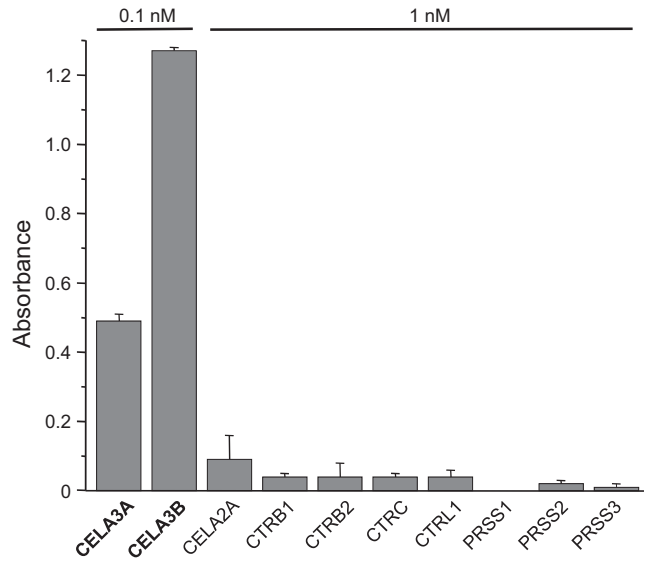


Fig. 2. Detection of human pancreatic serine proteinases by the ScheBo Pancreatic Elastase 1 Stool Test. Proteinases were expressed recombinantly and purified as described in MATERIALS AND METHODS. Chymotrypsin-like elastases 3A and 3B (CELA3A and CELA3B) were tested at 0.1 nM final concentration; all other proteinases were tested at 1 nM. For chymotrypsin-like enzyme 1 (CTRL1), conditioned medium diluted 1,000-fold was used. Values are means ± SD from 3 measurements. CTRB1, CTRB2, and CTRC, chymotrypsin B1, B2 and C, respectively; PRSS1, PRSS2, and PRSS3, human trypsin isoforms.

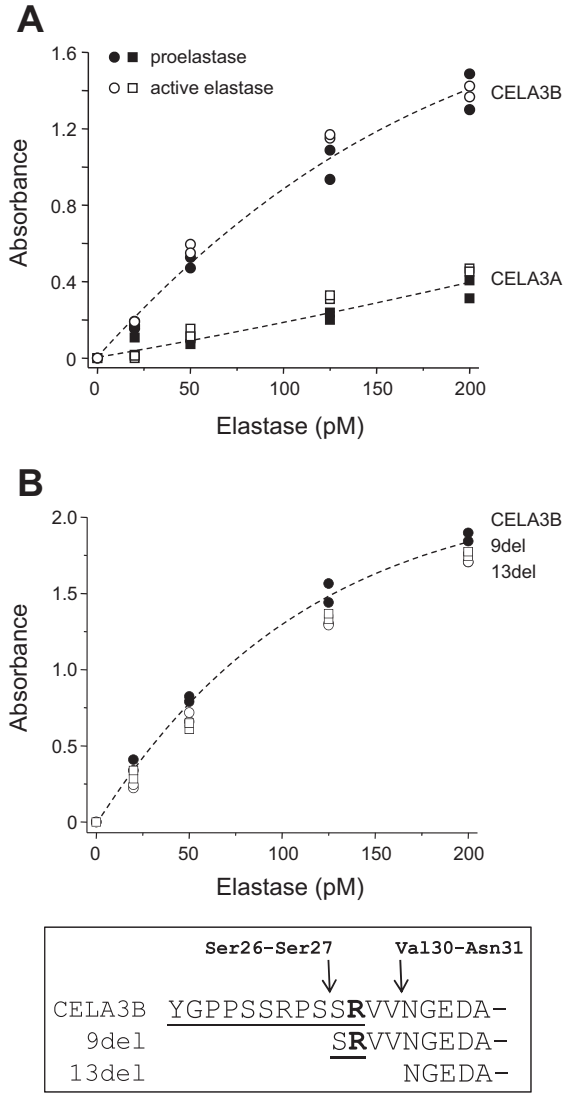


Fig. 3. Detection of CELA3 isoforms by the ScheBo Pancreatic Elastase 1 Stool Test. *A*: test performance on proelastases vs. active elastases at 20–200 pM. *B*: detection of autolyzed forms of CELA3B. *Inset*: autolytic cleavage sites in the NH_2-terminal part of CELA3B. The activation peptide is underlined. Assays were performed in duplicate, and both data points were plotted.

acid positions in CELA3B to the corresponding CELA3A amino acid. Positions where differences occurred in neighboring amino acids were mutated en bloc. Overall, 11 new CELA3B mutants were constructed and tested. For these qualitative screening experiments we used conditioned media of HEK 293T cells transfected with the mutant constructs. Remarkably, mutant E154K gave no ELISA signal, while all other mutants were robustly detected with some variations in signal strength (Fig. 5*B*). Since the mutations may alter secretion and enzyme activity of CELA3B, we also verified the expression of all mutants by SDS-PAGE and Coomassie staining (not shown) and by direct activity measurements after activation with trypsin. As shown in Fig. 5*C*, all mutants, including E154K, exhibited measurable elastase activity, and for the majority of mutants, activity was comparable to or even higher than that of the wild-type CELA3B. The higher activity of mutants S77R,S78D,W79L and D89N,R90L was due to greater amounts of elastase secreted into the conditioned medium (not shown), and this was also consistent with the stronger ELISA signal (Fig. 5*B*). Similarly, mutant A241G was secreted at higher levels (not shown) and produced a stronger ELISA signal, but this change was not obvious in the activity measurement, as this mutation decreases catalytic activity of CELA3B (25). This initial screen conclusively identified Glu^{154} in CELA3B as a major determinant of recognition by the ScheBo test.

To confirm the importance of Glu^{154}, we purified the E154K mutant and compared detection with that of wild-type CELA3B over a concentration range of 20–200 pM. No signal was obtained with the mutant (Fig. 6*A*). The ScheBo test uses a sandwich assay format with separate capturing and detection antibodies directed at different regions of the elastase molecules (Fig. 1). To ascertain whether the defect in the E154K mutant is at the level of capturing or detection, we eliminated the capturing step by immobilizing wild-type and mutant CELA3B to nickel plates (Ni-NTA HisSorb plate, Qiagen, Valencia, CA) via their His tag. Under these assay conditions, both elastase forms were detected comparably, indicating that mutation E154K interferes with the capturing step in the ELISA protocol (Fig. 6*B*). Finally, structural modeling indicated that Glu^{154} is located on the surface of CELA3B far from the active site (Fig. 6*C*).

Mutations of Lys^{154} and nearby Arg^{179} improve detection of CELA3A. The experiments presented above strongly indicate that detection of CELA3A should be improved by changing the Lys^{154} residue to Glu. However, we were surprised to find no improvement in detection over wild-type CELA3A when the CELA3A mutant K154E was purified and tested (Fig. 7*A*). To explain these puzzling observations, we speculated that the close proximity of another divergent amino acid interferes with the recognition of Glu^{154} in the CELA3A K154E mutant. Inspection of the CELA3B structural model indicated that amino acid 179, which is Arg in CELA3A and Leu in CELA3B, might be important in this regard (Fig. 6*C*). Indeed, when mutations K154E and R179L were introduced simultaneously in CELA3A, the strength of the ELISA signal was increased by ~2.5-fold and approximated that of CELA3B (Fig. 7*B*).

DISCUSSION

In the present study we evaluated the isoform specificity of the ScheBo Pancreatic Elastase 1 Stool Test. The clinical utility of the fecal elastase test in the evaluation of pancreatic insufficiency has been well established, and it has become widely used for routine indirect testing of pancreatic function (7, 8, 12, 15, 18-20, 23, 30, 32). Although limitations were also indicated by some studies (1, 20), the test gained popularity because it is noninvasive, relatively rapid, unaffected by pancreatic enzyme replacement therapy, and clearly superior to the previously used indirect test that measured fecal chymotrypsin activity. It is somewhat surprising that the exact molecular targets of this ELISA have not been characterized; it has been unclear which elastase isoform(s) is detected by the test and whether other homologous pancreatic proteinases interfere with the assay. The misnomer used in the test's commercial name adds to the uncertainty as to what exactly it measures. As discussed in the introduction, CELA1 is not expressed in the human pancreas, and, in all likelihood, the test's name

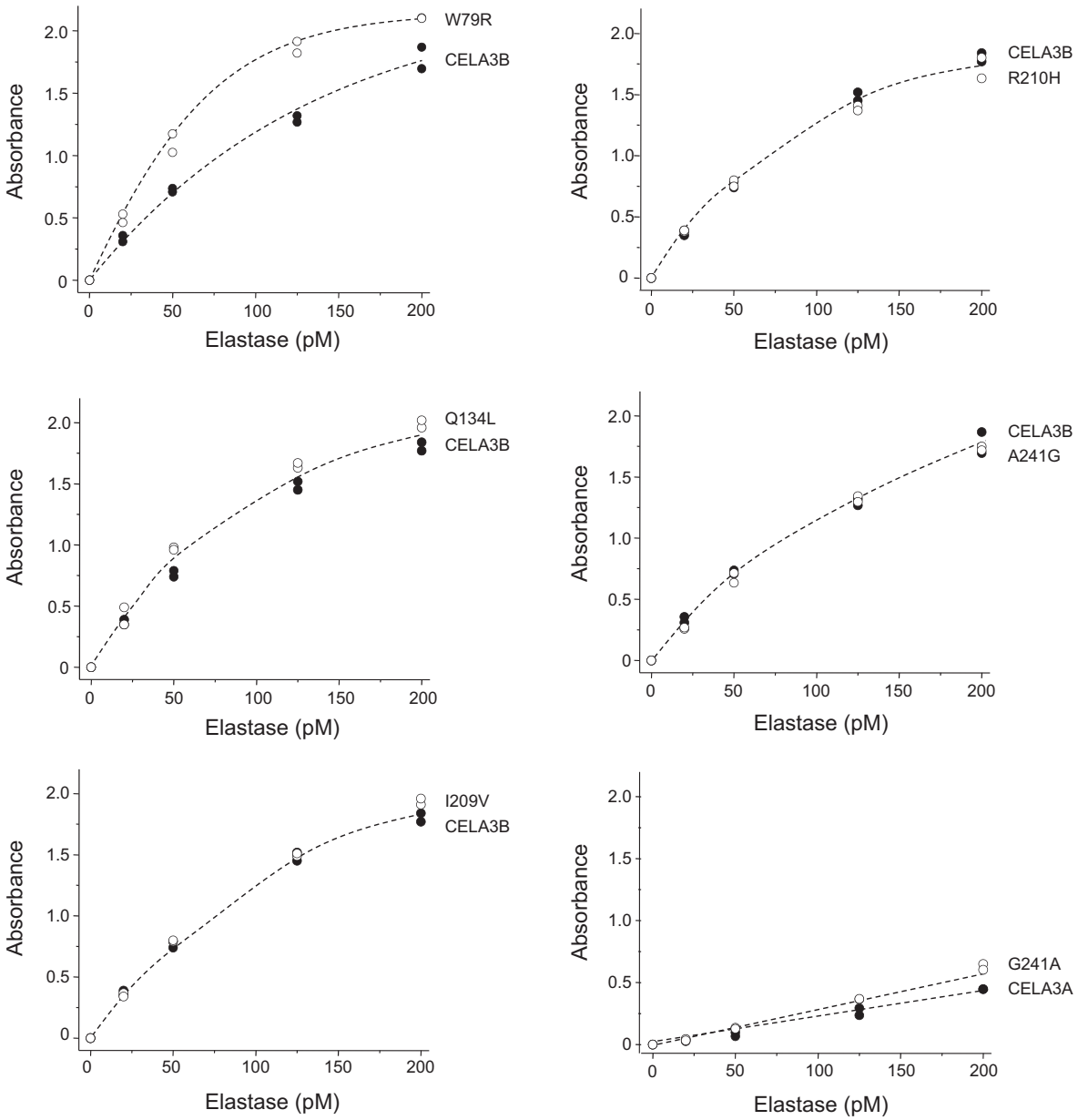
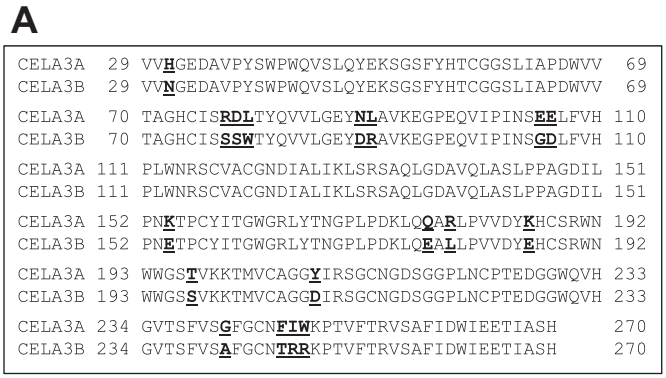


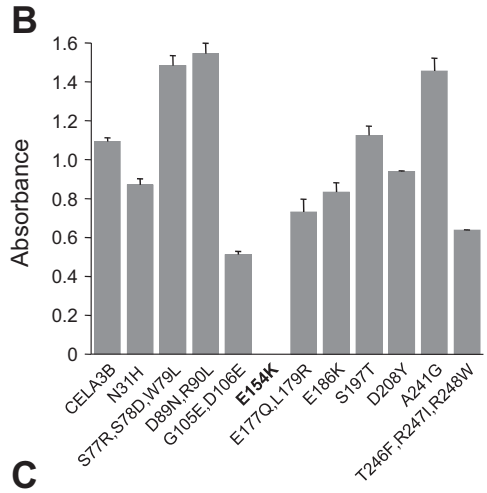
Fig. 4. Detection of naturally occurring CELA3A and CELA3B variants by the ScheBo Pancreatic Elastase 1 Stool Test. Wild-type CELA3A, CELA3B, and the indicated variants were purified and assayed at 20–200 pM. Assays were performed in duplicate, and both data points were plotted.

refers to one of the several historic names used for CELA3B in the article published by Largman et al. in 1976 (17). Thus we expected that the test would detect CELA3B; yet two vexing questions remained. *1*) Was the 92%-identical CELA3A isoform equally well detected? *2*) Did natural genetic variants of CELA3A and CELA3B affect test performance? Taking advantage of developments in the genomic sequencing and annotation of human digestive enzymes, we used well-defined recombinant preparations of human elastases, chymotrypsins, and trypsins to characterize the specificity of the ScheBo test.

Our findings confirmed that the primary target of the test is CELA3B; however, CELA3A, with a signal strength nearly fourfold weaker than that of CELA3B, was also detected. Importantly, CELA2A, chymotrypsins CTRB1, CTRB2, CTRC, and CTRL1, and trypsins PRSS1, PRSS2, and PRSS3 produced minimal or no signal in this ELISA. In 1984, Sziegoleit measured CELA3B levels as 4–6% of total pancreatic juice protein (36), and other studies indicated that mRNA and protein levels for the two CELA3 isoforms are grossly comparable (31, 42). However, how the expression of CELA3A and CELA3B in the pancreas might vary under different physiological and pathological conditions is unknown. It is intriguing to speculate that test performance might be affected by changes in the CELA3 isoform ratio. For this reason, development of a test that specifically detects CELA3B without cross-reactivity with CELA3A could potentially offer a better diagnostic tool.

Elastases are secreted as inactive precursors (zymogens) by the pancreas, and these proelastases become activated in the





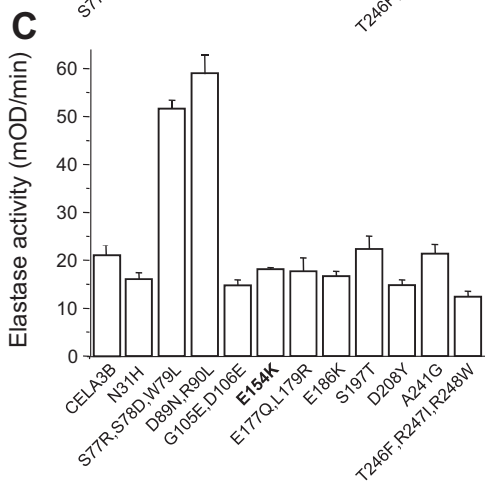
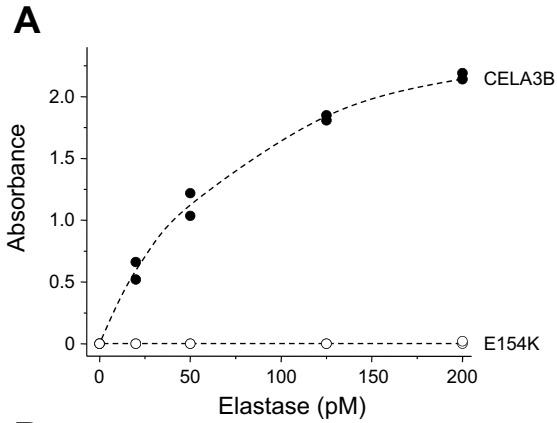
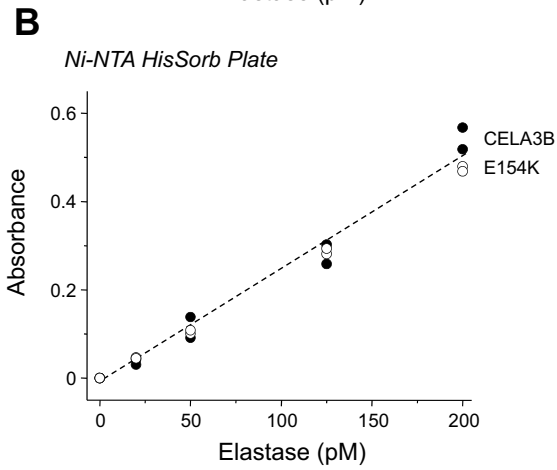


Fig. 5. Effect of amino acid differences between CELA3A and CELA3B on detection by the ScheBo Pancreatic Elastase 1 Stool Test. *A*: alignment of the amino acid sequences of the 2 human CELA3 isoforms. Active enzymes are shown; numbering starts with the initiator methionine of the pre-proelastases. Differences are underlined and boldface. *B*: ELISA of CELA3B mutants carrying substitutions with the corresponding CELA3A amino acids. HEK 293T cells were transfected with the indicated constructs, and conditioned medium was collected after 48 h. Elastase was assayed using 1,000-fold-diluted conditioned medium by the ScheBo test. *C*: elastase activity of CELA3B mutants in the conditioned medium after activation with trypsin. Values are means ± SD from 3 experiments. mOD, milli-optical density units.





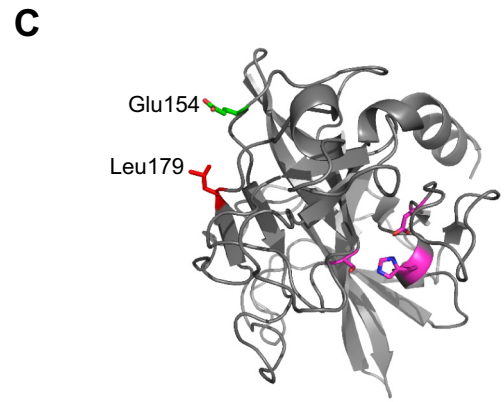


Fig. 6. Detection of CELA3B mutant E154K by the ScheBo Pancreatic Elastase 1 Stool Test. *A*: purified wild-type and E154K mutant CELA3B were assayed at 20–200 pM. *B*: purified wild-type and E154K mutant CELA3B were immobilized through their COOH-terminal His tags to Ni-NTA HisSorb plates (Qiagen) and detected with the biotinylated antibody-streptavidin-peroxidase complex from the ScheBo test. Assays were performed in duplicate, and both data points were plotted. *C*: structural model of human CELA3B indicating the positions of Glu^{154} (green) and nearby Leu^{179} (red). Also shown for reference are residues of the catalytic triad (magenta). Structural model for active CELA3B was generated by the SWISS-MODEL protein structure homology-modeling server (2) with porcine elastase used as template (PDB file 3UOU). Image was rendered with PyMOL (Schrödinger, Cambridge, MA).

duodenum by trypsin. Proelastases can also suffer autolysis due to their intrinsic zymogen activity or by active elastases (33). The most prominent autolytic cleavage takes place at the Val^{30}-Asn^{31} peptide bond in CELA3B (and, to a lesser extent,

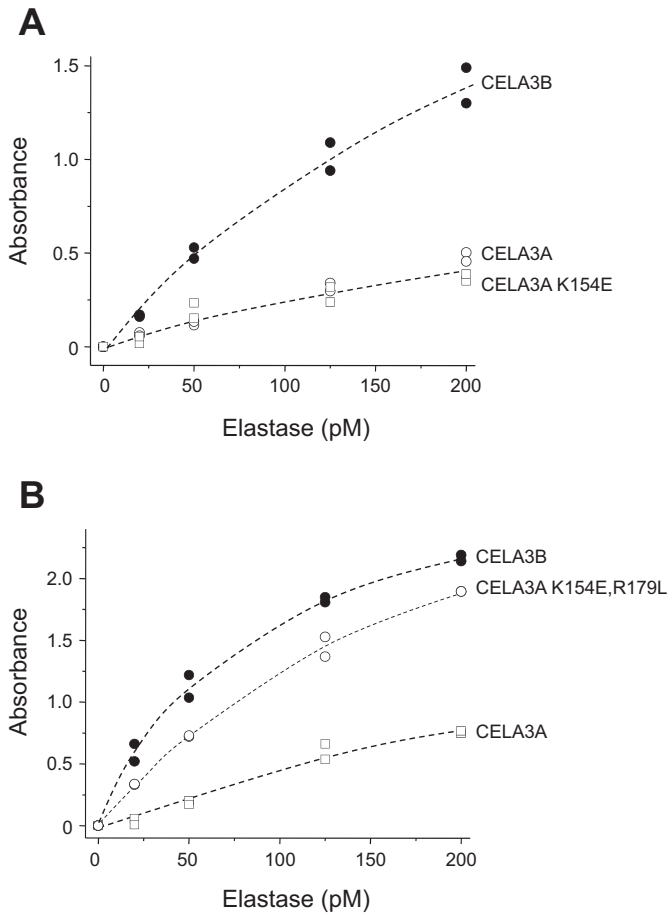


Fig. 7. Detection of CELA3A mutant K154E (*A*) and double mutant K154E,R179L (*B*) by the ScheBo Pancreatic Elastase 1 Stool Test. Wild-type CELA3A, CELA3B, and the indicated CELA3A variants were purified and assayed at 20–200 pM. Assays were performed in duplicate, and both data points were plotted.

at the Val^{30}-His^{31} peptide bond in CELA3A), resulting in a catalytically inactive elastase species (11, 33). We compared test performance for all molecular forms of CELA3B and found no difference in detection efficiency, indicating that test results are unaffected by the activation state or the degree of autolysis of secreted elastases.

Another potentially confounding factor is the occurrence of natural genetic variants of the CELA3 isoforms that might demonstrate an ELISA reaction different from that of the wild-type targets. The test is based on the binding of elastase to two monoclonal antibodies (Fig. 1), and genetic variants can alter the surface epitopes where these antibodies bind, resulting in altered detection efficiency. We characterized the effect on test performance of all genetic variants of CELA3A and CELA3B that occur at or above 1% frequency in the population and found no clinically meaningful changes. Only one variant, CELA3B W79R, exhibited increased detection, by ~1.4-fold, which should be inconsequential in heterozygous carriers. Our studies do not rule out the possibility that rare or private genetic variants in certain patients may interfere with the test; however, for the large majority of the population, the ScheBo test should not be affected by common genetic variants in the CELA3 isoforms.

We performed limited epitope mapping to identify amino acids that are responsible for the preferential detection of CELA3B over CELA3A by the ScheBo test. We identified Glu^{154} in CELA3B, which is Lys in CELA3A, as a key determinant of recognition by the capturing monoclonal antibody. Mutation E154K in CELA3B abolished detection by ELISA. It was surprising, however, that the opposite mutation K154E in CELA3A did not improve detection by the test, and simultaneous mutation of the nearby Arg^{179} to Leu (R179L) was required to achieve signal levels that were comparable to those of CELA3B.

Position 154 is located within a potential *N*-glycosylation site, and we considered the possibility that the side chain of amino acid 154 may alter glycosylation and, thereby, affect antibody recognition. *N*-linked glycosylation occurs on Asn residues in Asn-X_{aa}-Ser/Thr sequons, where X_{aa} can be any amino acid except Pro. Importantly, however, if the amino acid following the sequon is Pro, *N*-glycosylation is inhibited (22). In CELA3A the sequon Asn^{153}-Lys^{154}-Thr^{155} and in CELA3B the sequon Asn^{153}-Glu^{154}-Thr^{155} are followed by Pro^{156}, indicating that Asn^{153} is unlikely to undergo glycosylation in either isoform. This notion is consistent with earlier studies that characterized the glycosylation of CELA3B and found a single *N*-glycosylation site at Asn^{114} (45). Finally, although data are not shown, we observed no change in detection of CELA3A and CELA3B by the ScheBo test after treatment with peptide-*N*-glycosidase F.

In addition to the ScheBo test, which utilizes monoclonal antibodies, another clinical test developed and commercialized by Bioserv Diagnostics (Rostock, Germany) is based on detection by polyclonal antibodies against elastase 1 (12, 13, 23, 30). A recent study characterized the isoform specificity of the Bioserv test and found that CELA3A is a target, while CELA2A is not detected; however, other pancreatic proteinases were not evaluated in a comprehensive manner (44).

In summary, we characterized the molecular targets of the ScheBo Pancreatic Elastase 1 Stool Test and demonstrated that it predominantly measures CELA3B but also detects CELA3A with lower efficiency. Other pancreatic proteinases or genetic variants of the CELA3 isoforms have no appreciable impact on test performance.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

A.Z.T., P.H., and M.S.-T. conceived and designed research; A.Z.T., A.S., and E.H. performed experiments; A.Z.T., E.H., and M.S.-T. analyzed data; A.Z.T., E.H., P.H., and M.S.-T. interpreted results of experiments; A.Z.T. and M.S.-T. prepared figures; A.Z.T. drafted manuscript; A.Z.T., A.S., E.H., P.H., and M.S.-T. edited and revised manuscript; A.Z.T., A.S., E.H., P.H., and M.S.-T. approved final version of manuscript.

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