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Detection of human elastase isoforms by the ScheBo pancreatic elastase 1 test

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Running head: Elastase test isoform specificity

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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. Here we demonstrated 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.

Keywords: pancreas, digestive proteinase, elastase, chronic pancreatitis, pancreatic insufficiency

ABSTRACT

Determination of fecal pancreatic elastase content by ELISA is a reliable, non-invasive 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 3A and 3B (CELA3A and CELA3B) while CELA2A was not detected. Inactive proelastases, active elastases and autolyzed forms were detected with identical efficiency. CELA3B gave approximately four times higher signal than CELA3A and we identified Glu154 in CELA3B as the critical determinant of detection. Common genetic variants of CELA3A and CELA3B had no effect on ELISA signal strength 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.

INTRODUCTION

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, 19, 20, 23, 30, 32, 44]. The chymotrypsin-like elastases (CELAs) are digestive serine proteinases secreted by the pancreas in multiple isoforms. CELA1 was first described by Hungarian scientists Baló and Banga (1949) as an enzyme activity in the pig pancreas capable of hydrolyzing insoluble elastin [3, 4]. Due to 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, Ser) and aliphatic (Ile, Leu, Met, 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) [5 and references therein]. Curiously, while the human CELA1 gene appears to be potentially functional, it is not expressed in the pancreas due to evolutionary mutations in its promoter and enhancer regions [26, 41]. A second pancreatic elastase (CELA2) was identified based on its ability to solubilize elastin [17, for a complete list of references see 5]. Unlike CELA1, this elastase exhibits chymotrypsin-like P1 specificity and prefers to cleave after aromatic (Tyr, Phe) and aliphatic (Leu, 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 about 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 as 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 appear to be similar to that of porcine CELA1, broadly directed toward aliphatic P1 side chains [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 as 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 studies starting in 1982, Sziegoleit and coworkers characterized a so-called cholesterol-binding protein with proteolytic activity, which eventually turned out 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 those of CELA2A. Spurred by the observation that CELA3B suffers no proteolytic degradation during intestinal transit and appears in the stool in high concentrations [36], 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, 19, 20, 23, 30, 32]. One of the most widely used assays is the Pancreatic Elastase 1 Stool Test by ScheBo Biotech AG (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 to what extent 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 never 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 as the minor variants.

Plasmid construction and mutagenesis. Expression plasmids for human elastases CELA2A, CELA3A, CELA3B and chymotrypsins CTRB1, CTRB2, CTRC and CTRL1 constructed in the pcDNA3.1(-) vector [33 and references therein] and for human trypsins PRSS1, PRSS2 and PRSS3 in the pTrapT7 vector were described previously [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 9 His residues at their C terminus was created by gene synthesis and cloned into the pcDNA3.1(-) plasmid using EcoRI and BamHI restriction sites. In the CELA3B-9del construct the N-terminal 9 amino acids from Tyr18 to Ser26 were deleted. In the CELA3B-13del construct the N-terminal 13 amino acids from Tyr18 to Val30 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 Medium (DMEM) supplemented with 10% fetal bovine serum, 4 mM glutamine and 1% penicillin/streptomycin, at 37 ^{o}C. Transfections were performed with 4 µg expression plasmid with 10 µL Lipofectamine 2000 in 2 mL DMEM. After overnight incubation cells were rinsed and covered with 2 mL 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 C-terminal His tags by nickel-affinity chromatography, as reported previously [33]. Human trypsinogens were expressed in *E. 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 using the following extinction coefficients, calculated with the web-based ProtParam tool (in $M^{-1}\cdot cm^{-1}$ units): CELA2A 73,505; CELA3A 76,025; CELA3B 74,535; CELA3B mutant W79R 69,035; CTRB1 and CTRB2 47,605; CTRC 64,565; PRSS1 37,525;

PRSS2 38,890; PRSS3 41,535. 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]. N-terminal sequencing of the purified proteins revealed that ~90% of the CELA3B-9del preparation contained the expected N terminus of Ser27 and minor contaminants with N-terminal amino acids of Asn31 (5%) and Glu33 (5%). The purified CELA3B-13del contained an approximately 50-50% mixture of two forms; one with the expected N terminus of Asn31 and another species with an N-terminal Glu33, 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. Enzymatic 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 0.1 M $CaCl_2$ and incubated with 100 nM human cationic trypsin at 37 ^{o}C for 30 min (final concentrations). Activated elastases (20 μL) were then mixed with 175 μL assay buffer (0.1 M Tris-HCl (pH 8.0), 1 mM $CaCl_2$, 0.05% Tween 20) and elastase activity was measured by adding 5 μL of 6 mM substrate. The increase in absorbance at 405 nm was followed for 5 min in a microplate reader at 22 ^{o}C. Rates of substrate cleavage were calculated from the linear portion of the curves and expressed in mOD/min units.

ELISA assays. Detection of human pancreatic proteinases by the ScheBo test was performed according to the manufacturer's instructions with the ready-to-use reagents supplied, as follows. 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 $CaCl_2$, 0.05% Tween 20) were added to the ELISA strips containing an immobilized anti-elastase antibody (Figure 1). As blank, 50 μL assay buffer was used. After 30 min incubation at 22 ^{o}C, the enzyme solutions were removed from the wells. The wells were rinsed three times for 2 min each with 250 μL wash buffer. An aliquot (50 μL) of a biotinylated anti-elastase antibody complexed with peroxidase-conjugated

164 streptavidin was added to the wells and incubated for 30 min in the dark at 22 ^{o}C. The antibody
165 solution was discarded and the wells were rinsed three times for 2 min each with 250 µL wash
166 buffer. For color development, 100 µL 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid)
167 (ABTS) peroxidase substrate solution was added and incubated in the dark or 20 min at 22 ^{o}C.
168 The reaction was terminated by adding 100 µL stop solution and incubating for 10 min. The dark
169 green ELISA signal (absorbance) was measured in a plate reader at 405 nm. All assays were
170 performed in duplicates. Data points plotted represent absorbance readings corrected for the
171 average of two blank values.
172

173 **RESULTS**

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

181 **Proelastases, active elastases and autolyzed elastase forms are measured with equal**
182 **efficacy.** To characterize the detection of CELA3A and CELA3B by the ScheBo test in a more
183 quantitative manner, we performed the ELISA assay using elastases over a concentration range
184 from 20 to 200 pM. As shown in Figure 3A, CELA3B was detected on average 4.3-fold better
185 (range 3.4 – 5.1-fold) than CELA3A over the concentration range tested. Identical signals were
186 obtained when proelastases were compared to active elastases indicating that the test measures
187 the zymogen and active forms with equal efficacy (Figure 3A). Autolyzed forms of CELA3B
188 missing either 9 amino acids (9del) or 13 amino acids (13del) from the N terminus also produced
189 ELISA signals identical to that of the intact CELA3B proelastase (Figure 3B). Since these results
190 indicate that the ScheBo test does not discriminate between proelastase, active elastase and
191 autolyzed elastase, in all subsequent experiments we used the proelastase forms of CELA3A and
192 CELA3B. In a control experiment we also ruled out the unlikely confounding effect of the His
193 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 to comparable levels into the conditioned medium of transfected cells and gave identical ELISA signals.

Effect of *CELA3A* and *CELA3B* genetic variants on the 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 NHLBI Exome Sequencing Project. When considering missense variants only with an allele frequency above 1%, we found one *CELA3A* variant (G241A) and five *CELA3B* variants (W79R, Q134L, I209V, R210H and A241G). See Table 1 in reference [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 the performance of the ScheBo test, we purified these six variants and tested their detection in the ELISA assay. With the exception of the CELA3B W79R variant, none of the variants had an appreciable effect on signal development (Figure 4). Variant W79R was detected on average 1.4-fold (range 1.2 – 1.5-fold) better than wild-type CELA3B over the concentration range tested. However, considering that in most carriers the variant is heterozygous, this difference should have no meaningful impact on the clinical interpretation of the ScheBo test results.

Glu154 in CELA3B is a critical determinant of recognition by the ScheBo test. CELA3A and CELA3B share 92% identity at the amino-acid level yet CELA3B is detected circa 4-fold better than CELA3A by the ELISA assay. To identify the reason for this difference, we aligned the two isoforms (Figure 5A) and then individually mutated all divergent amino-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 whatsoever while all other mutants were robustly detected with some variations in signal yield (Figure 5B). Since the mutations may alter secretion and enzymatic 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 Figure 5C, all mutants, including E154K, exhibited measurable elastase activity and for the majority of mutants activity was comparable or even higher than that of the wild-type CELA3B. The higher activity of mutants S77R,S78D,W79L and D89N,R90L was due to higher elastase

225 amounts secreted to the conditioned medium (not shown) and this was also consistent with the
226 stronger ELISA signal (Figure 5B). Similarly, mutant A241G was secreted to higher levels (not
227 shown) and produced a higher ELISA signal but this change was not obvious in the activity
228 measurement as this mutation decreases catalytic activity of CELA3B [25]. Taken together, this
229 initial screen conclusively identified Glu154 in CELA3B as a major determinant of recognition
230 by the ScheBo test.

231 To confirm the importance of Glu154, we purified the E154K mutant and compared
232 detection to wild-type CELA3B over a 20-200 pM concentration range. No signal was obtained
233 with the mutant (Figure 6A). The ScheBo test uses a sandwich assay format with separate
234 capturing and detection antibodies directed at different regions of the elastase molecules (see
235 Figure 1). To ascertain whether the defect with the E154K mutant is at the level of capturing or
236 detection, we eliminated the capturing step by immobilizing wild-type and mutant CELA3B to
237 nickel plates (Ni-NTA HisSorb plate, Qiagen, Valencia, CA) via their His-tag. Under these assay
238 conditions both elastase forms were detected comparably, indicating that mutation E154K
239 interferes with the capturing step in the ELISA protocol (Figure 6B). Finally, structural modeling
240 indicated that Glu154 is located on the surface of CELA3B far removed from the active site
241 (Figure 6C).

242 **Mutations of Lys154 and nearby Arg179 improve detection of CELA3A**. The experiments
243 presented above strongly indicated that detection of CELA3A should be improved by changing
244 the Lys154 residue to Glu. Surprisingly, however, when the CELA3A mutant K154E was
245 purified and tested, no improvement in detection was observed over wild-type CELA3A (Figure
246 7A). To explain these puzzling observations, we speculated that the presence of another
247 divergent amino-acid that lies in proximity interferes with the recognition of Glu154 in the
248 CELA3A K154E mutant. Inspection of the CELA3B structural model indicated that amino acid
249 179, which is Arg in CELA3A and Leu in CELA3B, might be important in this regard (see
250 Figure 6C). Indeed, when mutations K154E and R179L were introduced simultaneously in
251 CELA3A, the ELISA signal was increased by about 2.5-fold and approximated that of CELA3B
252 (Figure 7B).
253

254 **DISCUSSION**

255 In the present study, we evaluated the isoform specificity of the ScheBo Pancreatic Elastase 1
256 Stool Test. The clinical utility of the fecal elastase test in the evaluation of pancreatic
257 insufficiency has been well established and it has become widely used for routine indirect testing
258 of pancreatic function [7, 8, 12, 15, 18, 19, 20, 23, 30, 32]. Although limitations were also
259 indicated by some studies [1, 20], the test gained popularity because it is non-invasive, relatively
260 rapid, unaffected by pancreatic enzyme replacement therapy and clearly superior to the
261 previously used indirect test that measured fecal chymotrypsin activity. Somewhat surprisingly,
262 the exact molecular targets of this ELISA assay have not been characterized; it has been unclear
263 which elastase isoform(s) the test detects, and whether other homologous pancreatic proteinases
264 interfere with the assay. The misnomer used in the test's commercial name adds to the
265 uncertainty as to what exactly it measures. As discussed in the introduction of this paper, CELA1
266 is not expressed in the human pancreas and the test's name in all likelihood refers to one of the
267 several historic names used for CELA3B in the article published by Largman et al. in 1976 [17].
268 Thus, we expected the test would detect CELA3B; yet the vexing questions remained whether
269 the 92% identical CELA3A isoform was equally well detected and whether natural genetic
270 variants of CELA3A and CELA3B affected test performance. Taking advantage of developments
271 in the genomic sequencing and annotation of human digestive enzymes, we used well-defined
272 recombinant preparations of human elastases, chymotrypsins and trypsins to characterize the
273 specificity of the ScheBo test.
274 Our findings confirmed that the primary target of the test is CELA3B, however, CELA3A is
275 also detected, approximately 4-fold weaker than CELA3B. Importantly, CELA2A,
276 chymotrypsins CTRB1, CTRB2, CTRC, CTRL1 and trypsins PRSS1, PRSS2, PRSS3 produced
277 minimal or no signal in this ELISA assay. Sziegoleit (1984) measured CELA3B levels as 4-6%
278 of total juice protein [36] and other studies indicated that mRNA and protein levels for the two
279 CELA3 isoforms are grossly comparable [31, 42]. However, there is no information available on
280 how the expression of CELA3A and CELA3B in the pancreas might vary under different
281 physiological and pathological conditions. It is intriguing to speculate that test performance
282 might be affected by changes in the CELA3 isoform ratio. For this reason, development of a test
283 that specifically detects CELA3B without any cross-reactivity with CELA3A could potentially
284 offer a better diagnostic tool.

285 Elastases are secreted as inactive precursors (zymogens) by the pancreas and these
286 proelastases become activated in the duodenum by trypsin. Proelastases can also suffer autolysis
287 due to their intrinsic zymogen activity or by active elastases [33]. The most prominent autolytic
288 cleavage takes place at the Val30-Asn31 peptide bond in CELA3B (and to a lesser extent at the
289 Val30-His31 peptide bond in CELA3A) resulting in a catalytically inactive elastase species [11,
290 33]. We compared test performance on all molecular forms of CELA3B and found no difference
291 in detection efficiency, indicating that test results are unaffected by the activation state or the
292 degree of autolysis of secreted elastases.

293 Another potentially confounding factor is the occurrence of natural genetic variants of the
294 CELA3 isoforms that might react differently with the ELISA assay than the wild-type targets.
295 The test is based on the binding of elastase to two monoclonal antibodies (see Figure 1) and
296 genetic variants can alter the surface epitopes where these antibodies bind, resulting in altered
297 detection efficiency. We characterized the effect on test performance of all genetic variants of
298 CELA3A and CELA3B, which occur at or above 1% frequency in the population and found no
299 clinically meaningful changes. Only one variant, CELA3B W79R, exhibited increased detection
300 by approximately 1.4-fold, which should be inconsequential in heterozygous carriers. Our studies
301 do not rule out the possibility that rare or private genetic variants in certain patients may interfere
302 with the test, however, for the large majority of the population the ScheBo test should not be
303 affected by common genetic variants in the CELA3 isoforms.

304 We performed limited epitope mapping to identify amino-acids that are responsible for the
305 preferential detection of CELA3B over CELA3A by the ScheBo test. We identified Glu154 in
306 CELA3B, which is Lys in CELA3A, as a key determinant of recognition by the capturing
307 monoclonal antibody. Mutation E154K in CELA3B abolished detection by the ELISA assay.
308 Surprisingly, however, the opposite mutation K154E in CELA3A did not improve detection by
309 the test and simultaneous mutation of the nearby Arg179 to Leu (R179L) was required to achieve
310 signal levels that were comparable to those of CELA3B.

311 Position 154 is located within a potential N-glycosylation site and we considered the
312 possibility that the side-chain of amino-acid 154 may alter glycosylation and thereby affect
313 antibody recognition. N-linked glycosylation occurs on Asn residues in Asn-Xaa-Ser/Thr
314 sequons where Xaa can be any amino acid except for Pro. Importantly, however, if the amino
315 acid following the sequon is a Pro, N-glycosylation is inhibited [22]. In CELA3A the sequon

316 Asn153-Lys154-Thr155 and in CELA3B the sequon Asn153-Glu154-Thr155 are followed by
317 Pro156, indicating that Asn153 is unlikely to undergo glycosylation in either isoform. This
318 notion is consistent with earlier studies which characterized the glycosylation of CELA3B and
319 found a single N-glycosylation site at Asn114 [45]. Finally, although data are not shown, we
320 observed no change in the detection of CELA3A and CELA3B by the ScheBo test after
321 treatment with peptide-*N*-glycosidase F (PNGase F).

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

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

332

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337

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342

343 **DISCLOSURES**

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346

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LEGENDS TO FIGURES

Figure 1. Schematic diagram of elastase detection by the sandwich ELISA method of the ScheBo Pancreatic Elastase 1 Stool Test. See *Methods* for details.

Figure 2. Detection of human pancreatic serine proteinases by the ScheBo Pancreatic Elastase 1 Stool Test. Proteinases were expressed recombinantly and purified as described in *Methods*. CELA3A and CELA3B were tested at 0.1 nM final concentration whereas all other proteinases were tested at 1 nM concentration. For CTRL1, conditioned medium diluted 1000-fold was used. Mean values with SD from three measurements are shown.

Figure 3. Detection of CELA3 isoforms by the ScheBo Pancreatic Elastase 1 Stool Test. (**A**) Comparison of test performance on proelastases versus active elastases in the 20-200 pM concentration range. (**B**) Detection of autolyzed forms of CELA3B. The inset demonstrates the autolytic cleavage sites in the N-terminal part of CELA3B. Assays were performed in duplicate and both data points were plotted.

Figure 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 in the 20-200 pM concentration range. Assays were performed in duplicate and both data points were plotted.

Figure 5. The 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 two human CELA3 isoforms. The active enzymes are shown; numbering starts with the initiator methionine of the pre-proelastases. Differences are underlined and emboldened. (**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 1000-fold diluted conditioned medium by the ScheBo test. See *Methods* for further details. (C) Elastase activity of CELA3B mutants measured in the conditioned medium after activation with trypsin. See *Methods* for experimental details. Mean values with SD from three experiments are shown.

489

490 **Figure 6.** Detection of CELA3B mutant E154K by the ScheBo Pancreatic Elastase 1 Stool Test.
491 (**A**) Purified wild-type and E154K mutant CELA3B were assayed over the 20-200 pM
492 concentration range. (**B**) Purified wild-type and E154K mutant CELA3B were immobilized
493 through their C-terminal His-tags to Ni-NTA HisSorb Plates (Qiagen) and detected with the
494 biotinylated antibody–streptavidin–peroxidase complex form the ScheBo test. Assays were
495 performed in duplicate and both data points were plotted. (**C**) Structural model of human
496 CELA3B indicating the positions of Glu154 (green) and nearby Leu179 (red). Also shown for
497 reference are the residues of the catalytic triad (magenta). The structural model for active
498 CELA3B was generated by the SWISS-MODEL protein structure homology-modeling server [2]
499 using porcine elastase as template (PDB file 3UOU). The image was rendered with PyMOL
500 (Schrödinger, Cambridge, MA).

501

502 **Figure 7.** Detection of CELA3A mutant K154E and double mutant K154E,R179L by the
503 ScheBo Pancreatic Elastase 1 Stool Test. Wild-type CELA3A, CELA3B and the indicated
504 CELA3A variants were purified and assayed in the 20-200 pM concentration range. Assays were
505 performed in duplicate and both data points were plotted.

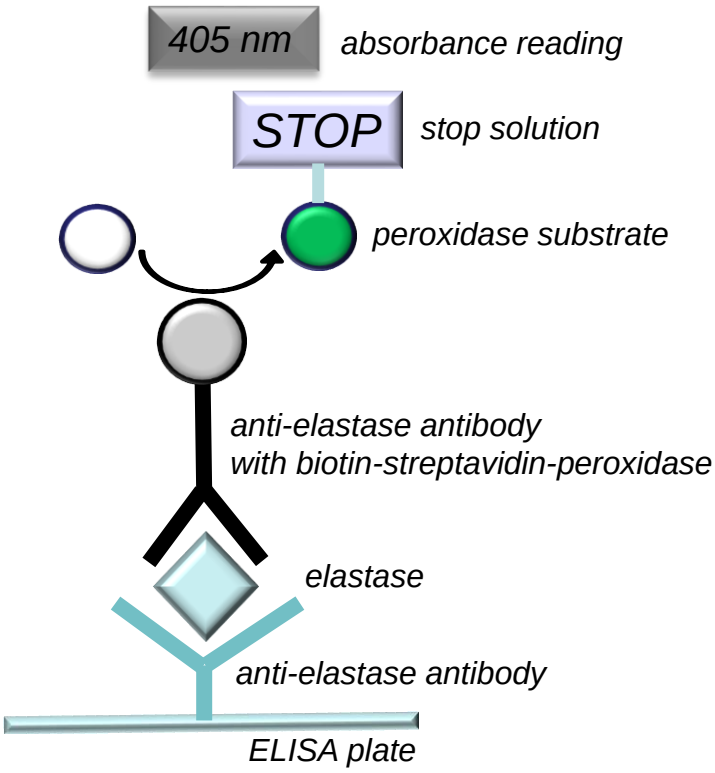
405 nm
absorbance reading
STOP
stop solution
peroxidase substrate
anti-elastase antibody
with biotin-streptavidin-peroxidase
elastase
anti-elastase antibody
ELISA plate

Figure 1

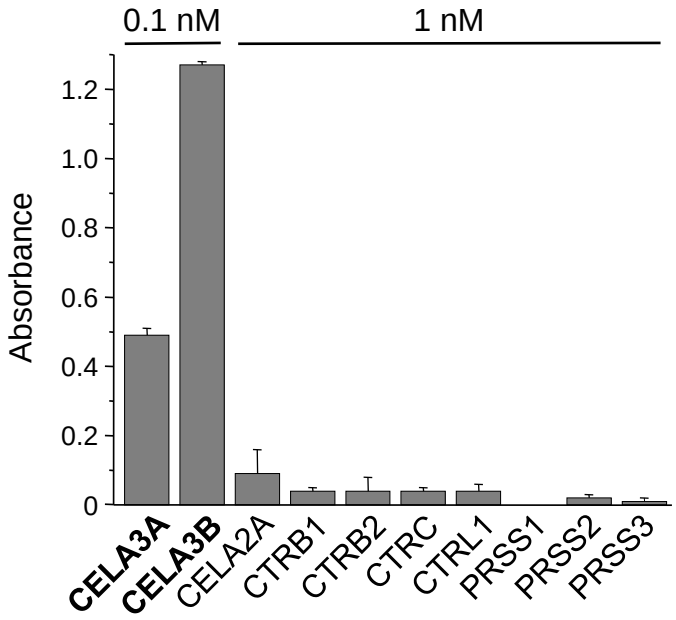
0.1 nM
1 nM
Absorbance
1.2
1.0
0.8
0.6
0.4
0.2
0
CELA3A
CELA3B
CELA2A
CTRB1
CTRB2
CTRC
CTRL1
PRSS1
PRSS2
PRSS3

Figure 2

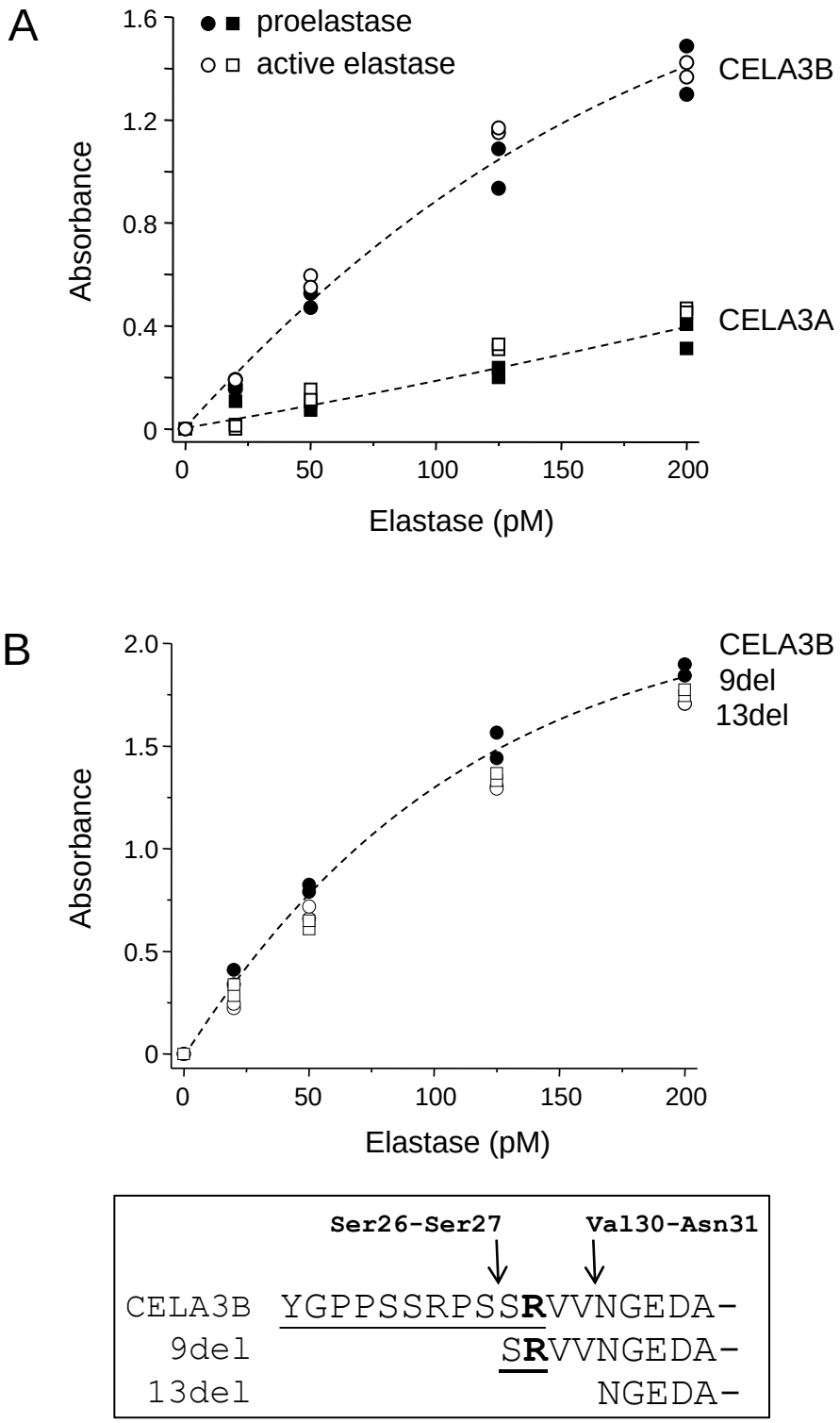
A
proelastase
active elastase
CELA3B
CELA3A
Absorbance
Elastase (pM)
B
CELA3B
9del
13del
Absorbance
Elastase (pM)
Ser26-Ser27
Val30-Asn31
CELA3B YGPPSSRPSSRVVNGEDA-
9del SRVVNGEDA-
13del NGEDA-

Figure 3

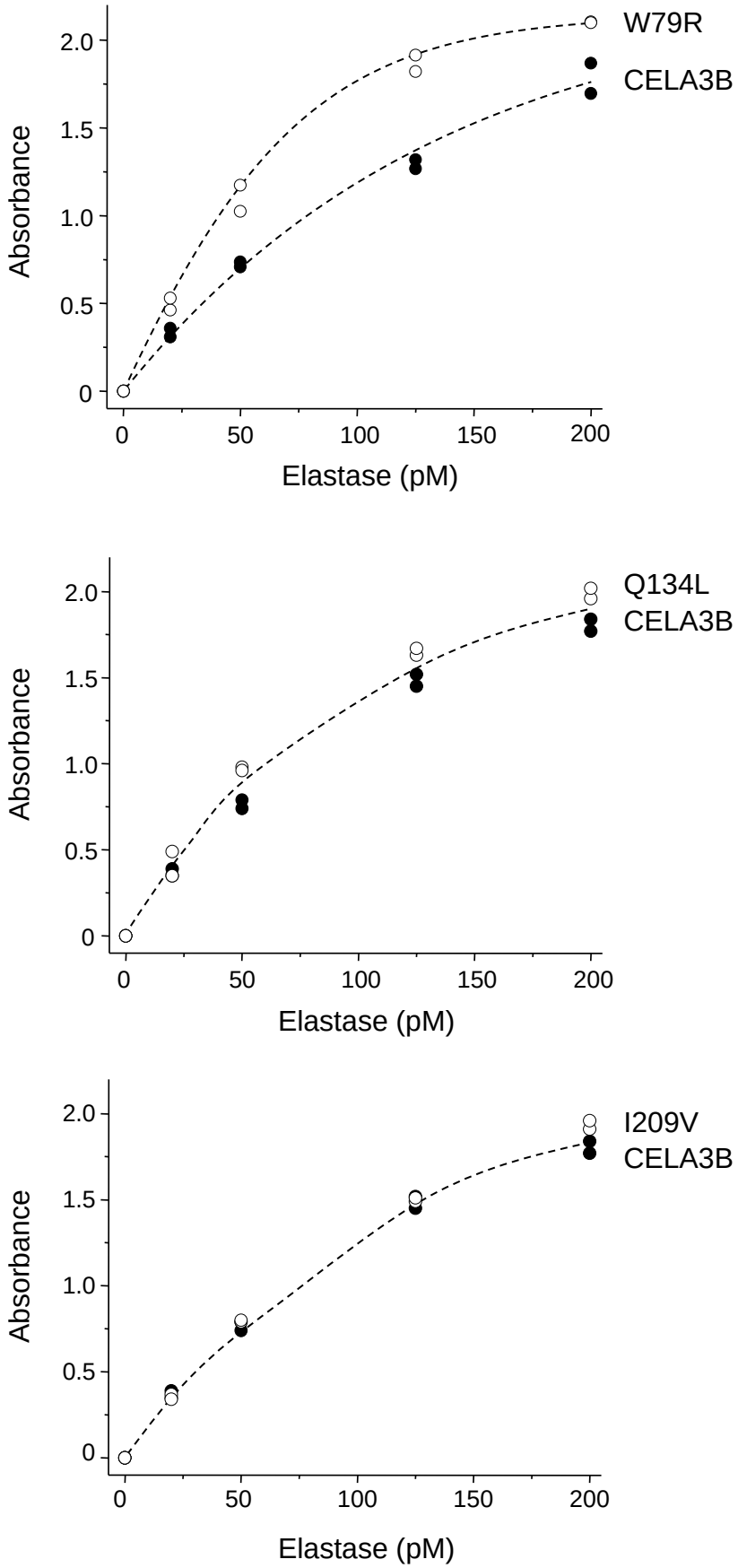
W79R
CELA3B
Absorbance
Elastase (pM)
Q134L
CELA3B
Absorbance
Elastase (pM)
I209V
CELA3B
Absorbance
Elastase (pM)

Figure 4

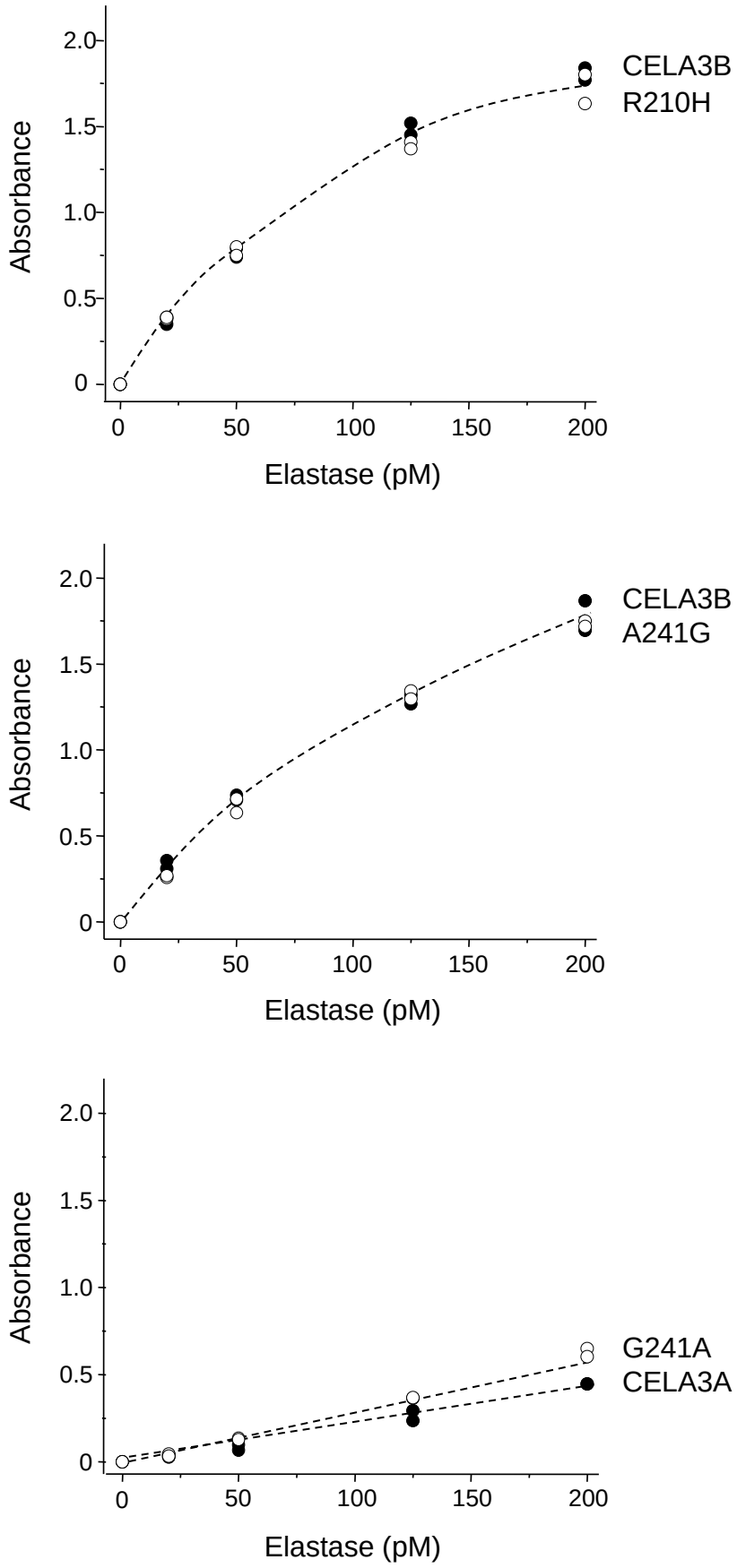

Figure 4 cont'd

A

```
CELA3A  29 VVHGEDAVPYSWPWQVSLQYEKSGSFYHTCGGSLIAPDWVV  69
CELA3B  29 VVNGEDAVPYSWPWQVSLQYEKSGSFYHTCGGSLIAPDWVV  69

CELA3A  70 TAGHCISRDLTYQVVLGEYNLAVKEGPEQVIPINSEELFVH 110
CELA3B  70 TAGHCISSSWTYQVVLGEYDRAVKEGPEQVIPINSGDLFVH 110

CELA3A 111 PLWNRSCVACGNDIALIKLSRSAQLGDAVQLASLPPAGDIL 151
CELA3B 111 PLWNRSCVACGNDIALIKLSRSAQLGDAVQLASLPPAGDIL 151

CELA3A 152 PNKTPCYITGWGRLYTNGPLPDKLQQARLPVVDYKHCSRWN 192
CELA3B 152 PNETPCYITGWGRLYTNGPLPDKLQEALLPVVDYEHCSRWN 192

CELA3A 193 WWGSTVKKTMVCAGGYIRSGCNGDSGGPLNCPTEDGGWQVH 233
CELA3B 193 WWGSSVKKTMVCAGGDIRSGCNGDSGGPLNCPTEDGGWQVH 233

CELA3A 234 GVTSFVSGFGCNFIWKPTVFTRVSAFIDWIEETIASH     270
CELA3B 234 GVTSFVSAFGCNTRRKPTVFTRVSAFIDWIEETIASH     270
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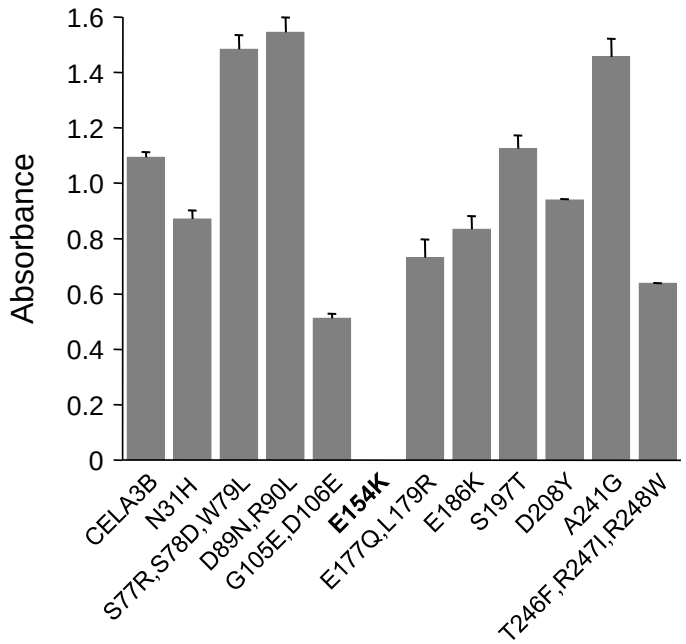
B



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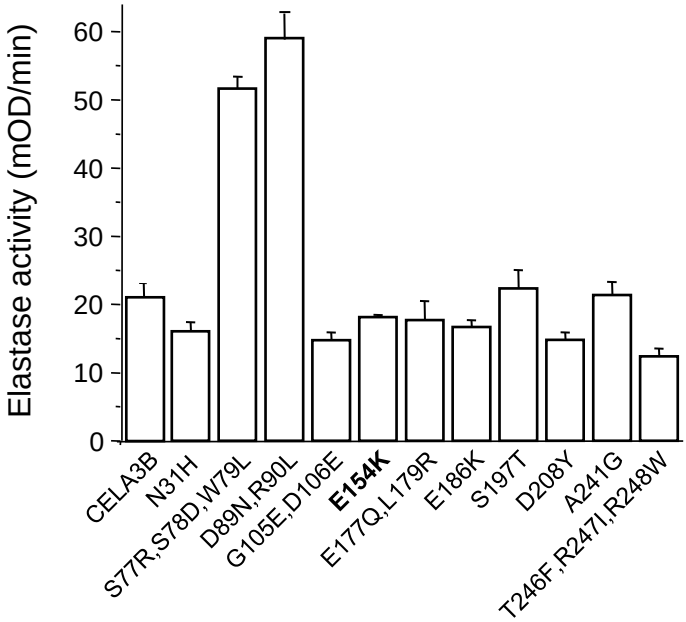
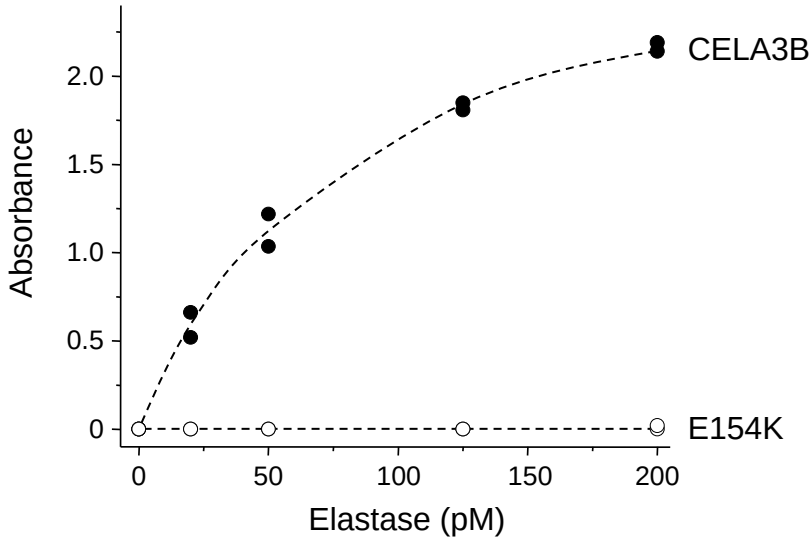


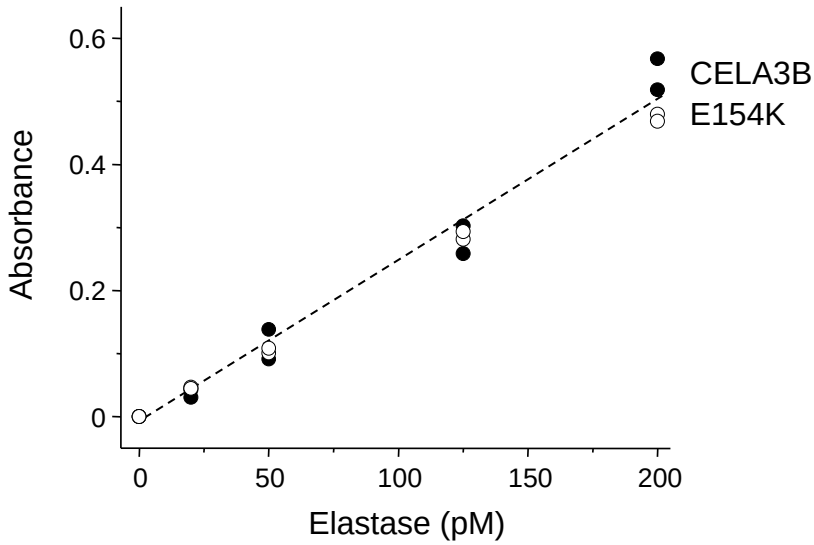
Figure 5

A



B

Ni-NTA HisSorb Plate



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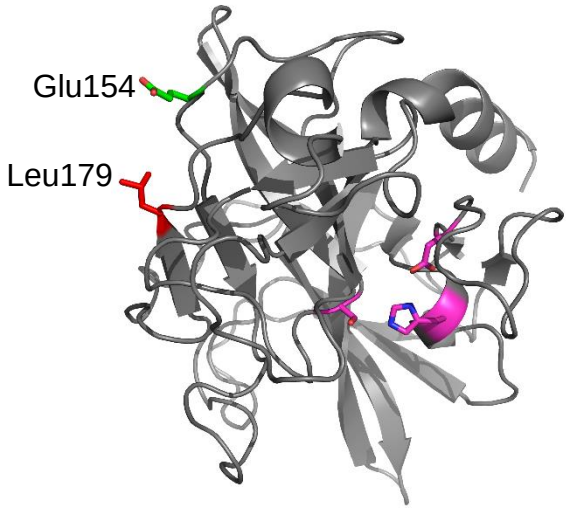

Figure 6

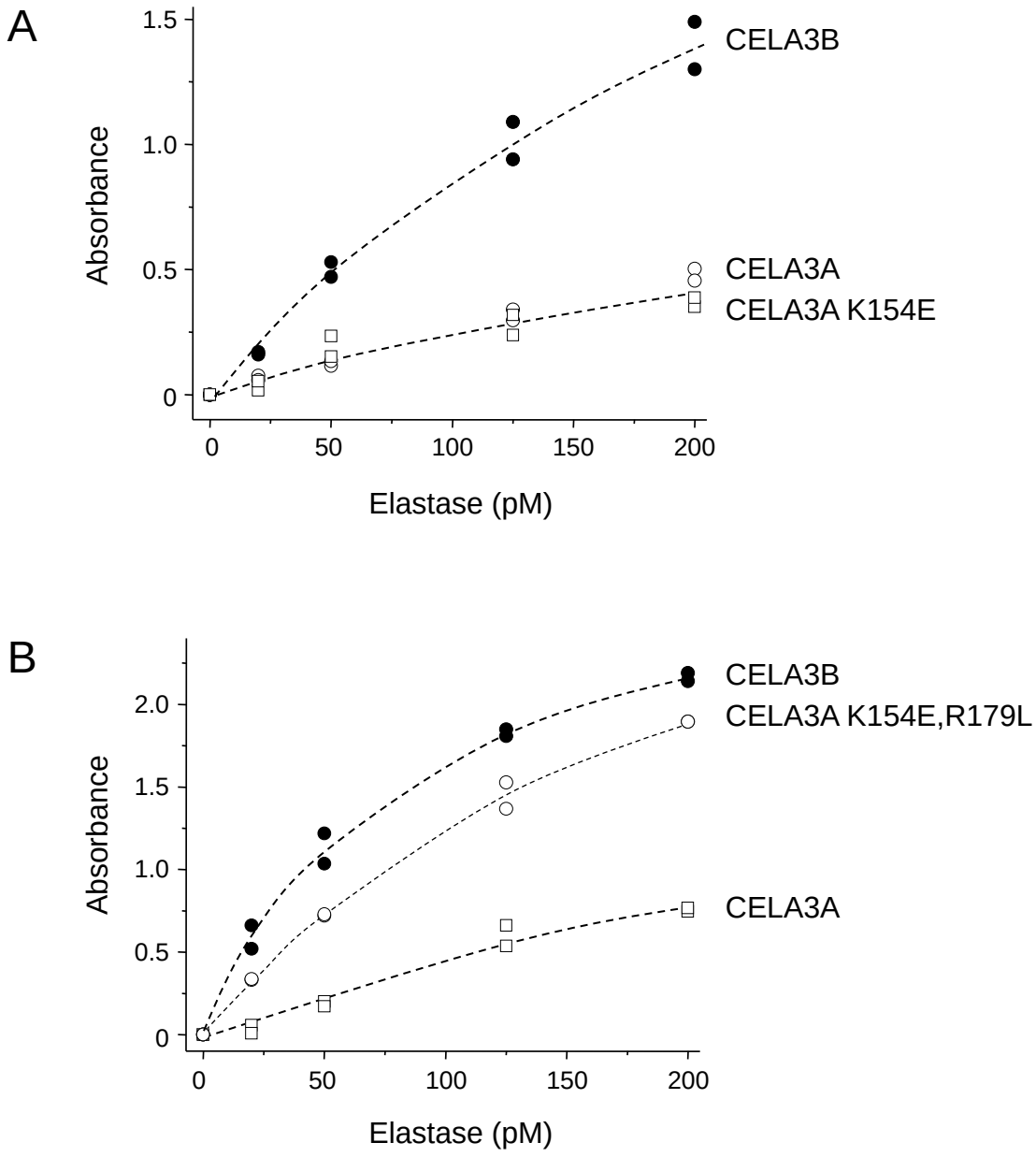
A
1.5
1.0
0.5
0
Absorbance
0
50
100
150
200
Elastase (pM)
CELA3B
CELA3A
CELA3A K154E
B
2.0
1.5
1.0
0.5
0
Absorbance
0
50
100
150
200
Elastase (pM)
CELA3B
CELA3A K154E,R179L
CELA3A

Figure 7