Stabilized variant of Streptomyces subtilisin inhibitor and its use in stabilizing subtilisin BPN′

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Protein protease inhibitors could potentially be used to stabilize proteases in commercial products such as liquid laundry detergents. However, many protein protease inhibitors are susceptible to hydrolysis inflicted by the protease. We have engineered Streptomyces subtilisin inhibitor (SSI) to resist proteolysis by adding an interchain disulfide bond and removing a subtilisin cleavage site at leucine 63. When these stabilizing changes were combined with changes to optimize the affinity for subtilisin, the resulting inhibitor provided complete protease stability for at least 5 months at 31°C in a subtilisin-containing liquid laundry detergent and allowed full recovery of the subtilisin activity upon the dilution that occurs in a North American washing machine.

Keywords: laundry/protease/SSI/Streptomyces subtilisin inhibitor

Introduction

Proteases are found in many commercial products, with the largest application currently being their use in laundry detergents (Houston, 1997). Proteases are known to undergo self-hydrolysis or autolysis. Such autolysis can limit the shelf-life of protease-containing products, especially if the product contains water, allowing protease activity during storage. Liquid laundry detergents are stabilized by small molecules, such as a combination of 1,2-propanediol and boric acid (Boyer and Farwick, 1994). These molecules minimize autolytic protease activity in the product yet dissociate on dilution of the product during usage, allowing the protease to provide cleaning performance. An alternative to using small molecules for controlling the protease activity would be to use an appropriate protein protease inhibitor. Protein protease inhibitors have a number of advantages, including the potential to be co-produced with the protease and to be used at lower concentrations than the inhibitors currently used.

Protein protease inhibitors must have certain attributes to be useful for stabilizing proteases in commercial products. First, these inhibitors must have the proper affinity for the protease to allow protease binding during product storage and dissociation upon product use. By the law of mass action, this ensures that a small but significant proportion of the protease will also be free in the stored product. Second, these inhibitors must be stable in the presence of surfactants and protease, potentially at the elevated temperatures of warehouses in summer.

We have developed variants of Streptomyces subtilisin inhibitor (SSI) for commercial stabilization of proteases. The engineering of SSI has been facilitated by a wealth of structural insight about the interaction between SSI and the protease subtilisin BPN′ (hereafter called BPN′). SSI was studied by a consortium of researchers, with their efforts giving rise to a book about SSI (Hiromi et al., 1985). SSI is a dimer of identical subunits, with each subunit interacting with the active site of the cognate protease (Tonmura et al., 1985). The inhibitor contains an extended region which fits into the active site of the protease, with Met73 of the inhibitor in a position to be attacked (Hirono et al., 1984). Changes in protease affinity have been documented for the replacement of Met73 by each possible amino acid (Kojima et al., 1991) and for the replacement of Met70 by three different amino acids (Kojima et al., 1990). Thermostable variants of SSI have also been described. The most dramatic effect was observed with a D83C change (Tamura et al., 1994), where an intersubunit disulfide bond provided a 14°C increase in denaturation temperature. Lesser thermal stability was achieved by increased hydrophilicity of the amino acid residue at position 73 (Tamura and Sturtevant, 1995a), an M103L change within the interior of SSI (Tamura and Sturtevant, 1995b) and a D83N change (Tamura et al., 1994).

In this paper, we show that a derivative of SSI can provide protease stability for at least 5 months at 31°C. Furthermore, the inhibitor’s affinity allows complete dissociation from the protease upon the 640-fold dilution of liquid laundry products that is typical for use in North American washing machines.

Materials and methods

DNA constructions

The expression plasmids are identical with the expression plasmids used for eglin (Qasim et al., 1997), except that the eglin-coding sequences were replaced by a synthetic gene encoding SSI. DNA was synthesized using an Applied Biosystems 381A synthesizer. The synthetic gene (Figure 1) was constructed by first carrying out the polymerase chain reaction (PCR) to amplify four pairs of overlapping single-stranded 60-base segments of DNA, one pair in each reaction. Two overlapping PCR products were then used in a second PCR, with one reaction generating the 5′ half of the gene and the other reaction generating the 3′ half. The 5′ PCR product was digested with the restriction enzymes EcoRI and PsI1, the latter site lying adjacent to the BamHI site (Figure 1). These digested PCR products were ligated to similarly digested pUC19 (New England Biolabs, Beverly, MA) and the ligation products were used to transform Escherichia coli strain MM294. The
appropriate clones, each containing one half of the synthetic SSI gene, were identified and confirmed by DNA sequencing. The entire synthetic SSI gene was subsequently assembled and cloned into the expression vector. The synthetic SSI gene encoded the same amino acid sequence as natural SSI (Obata et al., 1989) but was designed to be rich in AT residues, similar to Bacillus subtilis subtilisin. The following amino acid changes were made using the following oligonucleotides: M73P, 5' GAA GAC GTT ATG TGT CCC CCG GTT TAT GAT CCT GTA 3'; D83C, 5' GTA TTA CTG ACT GTT TGT; S98E, 5' GGA GGT GAC TTA AAC GCA ATA ACG CGT GTA TTA CTG ACT GTT TGT; A62K, 5' GGA GGT GAC TTA AAC AAA ATA ACA CGT GGT GAA GAC GTT; S98E, 5' TCT TAT GAA CGT TAT GGC GTA TAT GTG CAA GGT AAA CGT GTA TTC GAA AAT GAA TGT GAA ATG. Site-directed mutagenesis was performed as described, using the Kunkel method (Maniatis et al., 1982). Changes were confirmed by DNA sequence analysis and by mass spectrometry of the SSI derivative produced. The numbers for SSI residues correspond to the numbers of native SSI residues (Obata et al., 1989) without including the four additional amino acid residues encoded at the N-terminus (Figure 1). The B. subtilis host was PG632 (Saunders et al., 1991).

**Purification of SSI derivatives**

Strains were cultured in 25 ml of 2% tryptone, 2% yeast extract, 86 mM sodium chloride supplemented with 1.25 ml of 25% maltrin M250 (Grain Processing, Muscatine, IA), 1.25 ml of 2 N HEPES, pH 7.5, 200 µl of 10 mM MnCl₂ and 25 µl of 50 mg/ml kanamycin. Cultures were incubated overnight and the cells were removed by centrifugation. A 3 ml volume of 1 N HCl was added to the supernatant, bringing the pH to 4. The samples were incubated on ice for 30 min and then spun for 10 min at 12 000 r.p.m. The supernatant was collected and dialyzed against 4 l of 20 mM sodium acetate, pH 4. Precipitated material was removed by centrifugation. The supernatant was passed through an S Sepharose Fast Flow (Pharmacia) column in 20 mM sodium acetate, pH 4, washed with the same buffer and batch eluted with 0.5 M sodium chloride, 20 mM sodium acetate, pH 4. The samples were then dialyzed three times against 4 l of 10 mM Tris, pH 8.0 and finally once against 4 l of 1 mM Tris, pH 8.0. The samples were then filtered through a Millex GV filter (Millipore, Bedford, MA).

The concentration of SSI-A62K L63I M73P D83C S98E was determined using amino acid analysis. The concentration of other SSI derivatives was compared with SSI-A62K L63I M73P D83C S98E, using gel electrophoresis and comparing the intensity of the Coomassie Brilliant Blue-stained bands, using an Afga 4200e Scanner with BioRad PDQuest and Quantity One software. Using the same gel scanning technique, all SSI preparations were found to be >65% pure. Contaminants in all SSI preparations were similar, as judged by inspection of the gels, and consisted of a few minor bands and diffusely stained background material.

**Mass spectrometry**

To monitor the stability of SSI derivatives, samples were prepared with 2 mM Tris, pH 8, 2.5 µM BPN’ and 4.8 µM SSI derivatives. The samples were incubated at 31°C for 1 week. On-line liquid chromatography–electrospray mass spectrometry analysis of the SSI derivatives was carried out on a Q-TOF2 hybrid quadrupole time-of-flight mass spectrometer (Micromass, Manchester, UK). A capillary LC system (Waters, Milford, MA) was used for separation. Five microliters of each sample were loaded on a 0.32 mm × 15 cm Symmetry C18 column (5 µm, 300 A, Waters) using a gradient of 3–80% B over 20 min at a flow rate of 5 µl/min. Solvent A was water containing 0.02% trifluoroacetic acid (TFA) and solvent B was acetonitrile containing 0.02% TFA. A standard electrospray ionization interface was used for the LC–MS experiments, with the electrospray capillary voltage at 3 kV and cone at 35 V. The source and desolvation temperatures were maintained at 80 and 150°C, respectively, and the desolvation and cone gas flow rates were kept at 300 and 50 l/h, respectively. Mass spectra were recorded from mass 300–5000 Da every 2 s with a resolution of 10 000 (FWHM).

**Stability tests**

Stability tests were carried out at 31°C in a total volume of 400 µl, with 2.5 µM subtilisin BPN’, 0.2% sodium dodecyl
ethoxy sulfonate, 100 mM Tris, pH 8.0 and the SSI derivatives. To measure protease activity, a portion of the sample was diluted 64-fold into 50 mM Tris, pH 8.0. These diluted samples were assayed by the addition of succinyl-Ala-Ala-Pro-Phe-p-nitroaniline (Bachem) to a final concentration of 320 μM, and the reaction was monitored at 410 nm using a 1 cm cuvette in a Beckman DU-70 spectrophotometer. One unit of protease causes a rise of 1 optical density unit per minute. As a measure of the amount of free protease in the storage tube for each stability test, substrate was added directly to a storage tube and protease activity was monitored.

For stability tests in liquid Tide detergent (Correa et al., 2003) without protease stabilizing agents, 330 μl of a borax-free Tide formulation were used. Where indicated, the samples also contained 10 μM BPN’, 25 μg/μl Lipolase (Novozymes, Bagsvard, Denmark), 30 μM SSI-A62K L63I M73P D83C S98E and enough water to bring the sample volume to 400 μl. For lipase assays, a portion (10 μl) of the stability test sample was added to 90 μl of water and 10 μl of the dilution was assayed with 990 μl of assay buffer (26 mM Tris, pH 8.0, 1 mM CaCl2, 1% Tide and 200 μM p-nitrophenyl caprylate). The reaction rate was measured, with the background subtracted of a control reaction with no added lipase. For measuring protease activity, the 10-fold dilution, already described, was further diluted by mixing 15.6 μl of the dilution with 975 μl of the dilution with 975 μl of 50 mM Tris, pH 8.0. This sample was mixed with 320 μM succinyl-Ala-Ala-Pro-Phe-p-nitroaniline and the reaction rate was measured.

**Gel electrophoresis**

Part (90 μl) of the stability test sample was precipitated with 10% trichloroacetic acid (TCA) and suspended in 2x sample prep buffer (Novex) with 1% β-mercaptoethanol and 15 mM Tris base. Samples (5 μl for day 0 and day 3 samples, 9 μl for day 9; except for SSI-A62K L63I M73P D83C S98E, where 7 μl was used for each time point) were separated by electrophoresis through a 10–20% polyacrylamide gel (Novex).

**Ki determinations**

Ki was determined by incubating the SSI derivative and 39 nM BPN’ in 50 mM Tris, pH 8.0 with 640 μM succinyl-Ala-Ala-Pro-Phe-p-nitroaniline. As a control, we measured the protease activity in the absence of inhibitor. Ki values were calculated according to Goldstein (1944).

**Computer modeling**

Using the interactions displayed in the crystal structure of the 2SIC entry (Takeuchi et al., 1991) from the PDB, visual inspection was done using PSSHOW (Swanson, 1995) on a Silicon Graphics workstation. Proposed mutations were superimposed on the backbone positions and manually optimized by rotating the appropriate bonds.

**Results**

Protease inhibitors can be unfit for product storage because the inhibitors are unstable in the presence of surfactants and protease. For example, with wild-type eglin and eglin variants with a variety of single amino acid substitutions at the active site (Qasim et al., 1997), we found no inhibitor that was stable for more than a few days under these conditions (data not shown). Wild-type SSI was stable in the presence of protease and surfactant, but the inhibitor bound so tightly that there was no detectable free protease upon dilution (not shown). We created an SSI-M73P variant of lowered affinity for the protease (Kojima et al., 1991), with potential utility in allowing protease activity upon dilution. However, this SSI variant was not sufficiently stable for product use. Protease and inhibitor instability was shown, using BPN’ and SSI M73P (Figure 2). In the absence of SSI-M73P, the protease was unstable with a half-life of about 1 day (Figure 2). In the presence of SSI-M73P, the protease was not fully active upon dilution, as shown by the amount of protease activity at the start of the stability test. However, at day three, the protease activity increased, presumably reflecting a loss of inhibitor (see Figure 2).

![Graph](https://example.com/graph.png)

**Fig. 2.** Stability of protease BPN’ in the presence of different protease inhibitors. Starting protein concentration: BPN’, 2.5 μM; SSI-M73P, 8 μM; SSI-M73P D83C, 10 μM; SSI-L63I M73P D83C, 5 μM; SSI-A62K L63I M73P D83C S98E, 120 μM. Error bars represent one standard deviation.
below). The protease activity subsequently declined, presumably owing to autolysis, so that by the thirteenth day there was very little protease activity independent of whether the inhibitor had been added to the sample.

The SSI-M73P instability was also demonstrated by SDS gel electrophoresis (Figure 3A), with the inhibitor amount decreasing from day zero to day nine. Following the inhibitor loss, the protease disappeared, suggesting that the inhibitor was more susceptible to proteolysis than the protease was to autolysis. The inhibitor loss was due to the presence of the protease, as the inhibitor was stable in the absence of the protease (Figure 3A).

Upon gel electrophoresis, the SSI-M73P inhibitor and other inhibitors (see below) were separated into two species of similar molecular weight. Mass spectrometric analysis of all of these inhibitors indicated two species: one full length and one with Tyr7 at the N-terminus (not shown). These two forms probably corresponded to the two species separated by gel electrophoresis. In all of the BN’-containing samples, only the faster migrating species was visible at later times, suggesting that proteases can convert the higher molecular weight form to the lower molecular weight form.

**Inhibitor stabilization by D83C**

The SSI derivative can be stabilized by making site-directed changes. First, we introduced a covalent linkage between the two SSI monomer subunits, creating a covalently linked dimer. Such a change was already shown to increase the thermal stability of SSI (Tamura et al., 1994). Mass spectrometric analysis indicated that the D83C change led to covalent dimer formation. The mass of the SSI-M73P was 10 893, 1 Da less than the expected mass for monomer SSI with Tyr7 for its amino terminus (Figure 1). In contrast, the mass of SSI-M73P D83C was 21 762, 1 Da less than the expected mass for a dimer, linked by a covalent cystine bond and with Tyr7 for its N-terminus.

The D83C change stabilized the inhibitor to protease in the presence of surfactant (Figures 2 and 3A). The protease activity curve (Figure 2) revealed a slower rise in protease activity, with the peak protease activity occurring on the ninth day, indicating that the inhibitor was not degraded as quickly as was the M73P inhibitor. Such an interpretation was also indicated by the gel electrophoresis results (Figure 3A).

The stabilizing effect of the D83C inhibitor was not due to a difference in affinity for the protease, resulting in a change in the amount of free protease activity. The $K_i$ values for the M73P and M73P D83C products are 7.3 ($n = 2$, spread = 0.2) and 8 nM ($n = 2$, spread = 2.8), respectively, values that were not obviously different from each other. Furthermore, the amount of inhibitor used in the stability test was designed so that the amount of free protease was similar. Protease activity was measured directly under stability test conditions, with 0.12 ($n = 2$, spread = 0.03) protease activity units for the BPN’/SSI-M73P sample and 0.11 ($n = 2$, spread = 0.001) units for the BPN’/SSI-M73P D83C sample. Finally, the stability of protease activity was also monitored where each inhibitor was used at a 2:1 molar ratio to protease and SSI-M73P D83C again provided greater protease stability (not shown).

**Inhibitor stabilization by L63I**

Although the D83 change provided stability to SSI, that stability was not sufficient for product storage. To design an even more stable inhibitor, we used mass spectrometry to determine the site at which the protease cleaved SSI-M73P D83C. This experiment was carried out in the absence of surfactants, as the presence of surfactants made mass spectrometric analysis difficult.

After storage of SSI-M73P D83C in the presence of BPN’, the primary product had a molecular weight of 21 764, 1 Da more than the expected mass of the covalent dimer of subunits extending from Tyr7 to Phe113, the carboxy end (Figure 4). There was a smaller amount of a breakdown product, with the...
mass of a dimer of a Tyr7 to Phe113 subunit and a Thr64 to Phe113 subunit, suggesting that cleavage of SSI-M73P D83C occurred between Leu63 and Thr64. An identical length breakdown product was also observed in a stability test with a SSI-M73G D83C, and, in that case, Edman sequencing was used to confirm the cleavage site after Leu63 (data not shown). This breakdown product indicated that BPN\textsuperscript{0} cleaved SSI-M73P at a site other than position 73, at which SSI normally binds to the protease active site. We wanted to reduce the amount of interaction, especially hydrolysis, between BPN\textsuperscript{0} and the L63 region of SSI. In a P\textsubscript{1} position, leucine is a good substrate for BPN\textsuperscript{0} activity (Morihara and Tsuzuki, 1969; Estell et al., 1986). We used oligonucleotide-directed mutagenesis to replace Leu63 with isoleucine, known to be a poor P\textsubscript{1} substrate (Morihara and Tsuzuki, 1969; Lu et al., 1997), to reduce proteolysis with minimal change to the inhibitor structure and overall affinity for the BPN\textsuperscript{0}. This new inhibitor, SSI-L63I M73P D83C, was incubated with BPN\textsuperscript{0} in the absence of surfactant and the inhibitor stability was monitored by mass spectrometry. The new inhibitor was more stable, with no detectable cleavage adjacent to Ile63. There was a lesser amount of a new breakdown product observed (Figure 4), apparently the result of cleavage at a new site, between Thr64 and Arg65.

Stability test analysis also indicated the greater stability of the L63I-modified inhibitor (Figures 2 and 3). These stability tests were somewhat complicated by the higher affinity of the L63I-modified inhibitor for BPN\textsuperscript{0}. The $K_e$ value for SSI-L63I M73P D83C was 2.3 nM ($n = 3$, spread = 0.9), about 3-fold lower than the $K_e$ values for the other two inhibitors. For the stability test, the inhibitors were used in amounts that provided nearly equal amounts of free protease in the product. In a direct

Fig. 4. Mass spectra of SSI derivatives after storage at 31°C in the presence of BPN\textsuperscript{0}. (A) SSI-M73P D83C sample comprised a disulfide-linked dimer (YAP–FAF/ YAP–FAF, MW 21 764) (see Figure 1) and a breakdown product (YAP–FAF/TRG–FAF, MW 16 492). (B) SSI-L63I M73P D83C sample comprised a disulfide-linked dimer (YAP–FAF/YAP–FAF, MW 21 764) and a new breakdown product with the mass of YAP–FAF/RG–FAF, MW 16 391.
protease assay under stability test conditions, the BPN/SSI-L63I M73P D83C sample had 0.13 (n = 2, spread = 0.02) units of protease activity, similar to the other samples (see above). By monitoring protease activity over time, the SSI-L63I M73P D83C showed greater stability than the other inhibitors, with a broad peak of protease activity from days nine to 14, somewhat later than observed with SSI-M73P D83C (Figure 2). A more obvious demonstration of the increased stability with the L63I change is shown by gel electrophoresis of these stability test samples, where only the SSI-L63I M73P D83C was readily visible after 9 days (Figure 3).

**Enzyme stabilization in Tide**

For liquid laundry applications, the inhibitor must be of sufficiently low affinity to enable dissociation from the protease upon dilution into the washing machine yet sufficiently high affinity for protease binding in the product. All of the previously described SSI inhibitors are of too high affinity to provide full dissociation from the protease upon the 640-fold dilution of North American washing machines. Therefore, we made two additional changes to the inhibitor to tailor the affinity. Using computer modeling, we predicted that an S98E change would reduce affinity by generating a repulsion with E156 of BPN' (Mitsui, 1985). An A62K change was expected to have but a small effect on affinity, slightly stabilizing the complex by forming a salt bridge with D59 of the same SSI chain. The resulting inhibitor, SSI-A62K L63I M73P D83C S98E, bound to BPN' with a *K* of 160 ± 17 nM (n = 6), an affinity that seemed ideal.

SSI-A62K L63I M73P D83C S98E was stable under the conditions where the other SSI derivatives were tested (Figures 2 and 3B), at an equivalent amount of protease activity (0.15 ± 0.2 units, n = 3, spread = 0.04). Note that because of the lower affinity of this inhibitor, a larger inhibitor concentration (Figure 2) was required to provide this extent of protease inhibition.

SSI-A62K L63I M73P D83C S98E was tested for its stability in the presence of a Tide formula lacking borax, the protease stabilizing material normally used in this product (Figure 5A). The SSI-A62K L63I M73P D83C S98E allowed full dissociation of the protease upon dilution, as indicated by the equivalent protease activity, at the starting time, of samples regardless of whether the protease inhibitor was present. The inhibitor and protease were stable in Tide, as indicated by the complete retention of protease activity for 5 months.

As an additional indication of the effect of the protease inhibitor, we included a lipase in the stability test. In control experiments without protease inhibitor, the lipase was sensitive to BPN'-mediated proteolysis, with <15% of the lipase activity remaining after 8 days (Figure 5B). In a control experiment (not shown), the lipase activity was completely maintained for at least 3 months in the absence of protease, indicating that lipase loss was due to proteolysis. In the presence of SSI-A62K L63I M73P D83C S98E, the lipase was stabilized, with about half of the lipase activity still present after 50 days. This further indicated that the SSI1 variant had reduced the protease activity during product storage.

**Discussion**

Protein protease inhibitors can provide stability to proteases and other enzymes in liquid laundry products. However, the protein protease inhibitor must be stable in the presence of protease and surfactants. Furthermore, the inhibitor must have the correct affinity to bind protease sufficiently tightly in the product and dissociate upon dilution into the washing machine. We have created an SSI variant, A62K L63I M73P D83C S98E, that meet these criteria.

This inhibitor was the result of several changes directed at inhibitor stability and affinity for the protease. Stability was indicated using protease assays and gel electrophoresis. The D83C and L63I changes each provided greater stability to the protease in the presence of a surfactant. D83C was already known to increase thermal stability of SSI (Tamura et al., 1994). We hypothesize that the protease resistance was due to the disulfide bond providing a more rigid structure, with less thermal motion to expose protease-sensitive sites. The L63I change provided protease resistance by replacing a residue, Leu63, which was the target for cleavage by BPN'. The isoleucine residue is not well accommodated by the narrow mouth of the S1 site of BPN', potentially contacting the side chain of...
L125 and the amide nitrogen of G127 of BPN'. These steric constraints apparently prevent inhibitor cleavage at the L63I position, as there was no detectable cleavage at this site. However, a reduced amount of cleavage was detected at an adjacent site. This region of the inhibitor is fairly unstructured (Hirono et al., 1984) and part of the inhibitor's surface, making the region a likely candidate for protease action.

This is the first report of SSI engineered to show an increase in resistance to protease. In contrast, decreased protease resistance has been noted with SSI modifications. First, carboxypeptidase A was used to remove the C-terminal four amino acid residues of SSI and the truncated inhibitor was more susceptible to BPN' (Akasaka et al., 1985). Second, SSI degradation by BPN' was stimulated by the following genetic variants of SSI: an M73C substitution (Kojima et al., 1991), a W86H substitution (Tamura et al., 1991), a deletion of amino acid residues 64 and 66 (Kojima et al., 1993a) and removal of an intrasubunit disulfide bond between Cys71 and Cys101 (Kojima et al., 1993b).

The A62K, M73P and S98E changes combined to reduce the affinity of the inhibitor for the protease, allowing the complex to dissociate upon the 640-fold dilution common in North American washing machines. The greatest effect was due to the M73P change, predicted to induce a major twist to the backbone at the P1 site, reducing affinity for the protease. This change was known to lower the affinity for protease by SSI (Kojima et al., 1991; Masuda-Momma et al., 1993) and analogues of other inhibitors lead to reduced affinity (Lu et al., 1997; Qasim et al., 1997). The remaining loss of affinity was due to a combination of the S98E and A62K changes. As we have not individually tested the effects of these changes on affinity for BPN', we cannot conclude how much of the affinity change is due to the individual changes. However, we predict from the crystal structure (Hirono et al., 1984) that the major contribution of the two was from the S98E change. The S98E substitution has not been found among 20 similar protease inhibitors from Streptomyces and Streptoverticillium (Taguchi et al., 1997), as only serine, glycine and alanine are found at position 98. By contrast, position 62 exhibits greater diversity and Lys62 is found in three of these inhibitors.

The SSI-A62K L63I M73P D83C S98E bound the protease sufficiently tightly in the stability test sample, as indicated by the protection of not only the protease activity but also a lipase added to the sample. The SSI dissociated from the protease upon dilution, as shown by the full protease activity upon dilution of the stability test samples. This is the first report of a protein protease inhibitor shown to provide product stability and yet allow full dissociation upon product usage conditions. Having met these criteria for product performance, there remain additional criteria for commercialization, including inhibitor cost and safety profile.

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