A transgenic plant cell suspension culture was established as a versatile and efficient expression system for the subtilase SlSBT3 from tomato. The recombinant protease was purified to homogeneity from culture supernatants by fractionated ammonium sulfate precipitation, batch adsorption to cation exchange material, and anion exchange chromatography. Purified SlSBT3 was identified as a 79-kDa glycoprotein with both complex and paucimannosidic type glycan chains at Asn177, Asn203, Asn376, and an N-linked oligosaccharide side chain. The enzyme was fully active at 60 °C and showing highest activity at alkaline conditions with a maximum between pH 7.5 and 8.0. Substrate specificity of SlSBT3 was analyzed in detail, revealing their features were found to be required for SlSBT3 activity and, consequently, for prodomain processing and secretion.

The nine mammalian proprotein convertases (PCs)3 belong to the subtilase family of serine proteinases (family S8 within the subtilase family) (1), which is characterized by a catalytic triad of Asp, His, and Ser residues in an arrangement shared with subtilisins from Bacillus species (2). They are all involved in the processing of precursor proteins by limited proteolysis at highly specific sites. Seven of the nine PCs are related to yeast kexin and cleave their substrates at single or pairs of basic residues, predominantly on the carboxyl side of the motif (Arg/Lys)-Xn-Arg. Their substrates include polypeptide hormone precursors, growth factors, receptors, enzymes, and viral surface glycoproteins (3). The remaining two PCs, SKI-1 (subtilisin/kexin-isozyme-1)/S1P (site-1 protease) and NARC-1 (N-arginine dibasic convertase)/PCSK9, belong to the pyrolysin and proteinase K subfamilies of subtilases, respectively (4), and they are involved in precursor processing at nonbasic sites (3, 5, 6).

As compared with mammals, many more subtilases exist in higher plants, but their function is only just beginning to be elucidated. Although 15 subtilase genes have been characterized in tomato (7) and 63 in rice (8), most work focused on the 56 subtilases in Arabidopsis thaliana (9). Two of the Arabidopsis enzymes, AtSBT6.1 and AtSBT6.2, appear to be orthologs of other eukaryotic subtilases. Like SKI-1/S1P in mammals (5), the Arabidopsis subtilase AtSBT6.1 (syn. AtS1P) is involved in the stress-induced processing of a membrane-associated transcription factor, resulting in the activation of stress response gene expression. This transcription factor, bZIP17, is a predicted type II membrane protein with its basic leucine zipper (bZIP) domain on the cytoplasmic side and a canonical S1P cleavage site facing the luminal side of the endoplasmic reticulum (ER) membrane. In response to salt stress, bZIP17 is processed by Golgi-resident AtS1P. Consequently, the N-terminal fragment is released from the membrane, relocates to the nucleus, and activates the expression of stress response genes (10). This salt stress response in Arabidopsis is related to ER stress in mammals, where the accumulation of unfolded or misfolded proteins results in the transport of membrane-bound ATF6 (activating transcription factor 6) to the Golgi, where it is processed on its lumen-facing side by SKI-1/S1P (11). Similarly, in Arabidopsis, the role of ATF6 as a sensor/transducer of the unfolded protein response is adopted by bZIP28, which is likely to be

The nine mammalian proprotein convertases (PCs)3 belong to the subtilase family of serine proteinases (family S8 within

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The on-line version of this article (available at http://www.jbc.org) contains supplemental Methods, Table S1 and Figs. S1–S4.

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3 The abbreviations used are: PC, proprotein convertase; MALDI-TOF, matrix-assisted laser desorption/ionization-time of flight; MS, mass spectrometry; PA, protease-associated; TLCK, N α-tosyl-L-lysylchloromethylketone; TPCK, N α-tosyl-L-phenylalaninylchloromethylketone; bZIP, basic leucine zipper; ER, endoplasmic reticulum; MES, 4-morpholineethanesulfonic acid; PNGase F, peptide:N-glycosidase F; Endo H, endoglycosidase H; E3, ubiquitin-protein isopeptide ligase.
Biochemical Characterization of SBT3 in Tomato

In contrast to mammalian PCs and bacterial subtilisins, very little is known about the maturation and sorting of plant subtilases and the determinants of substrate specificity. The similarity between the catalytic domains notwithstanding, simple extrapolation from our knowledge of animal PCs may be misleading, since considerable differences exist within both the N- and the C-terminal extensions of plant subtilases. Another distinguishing feature of plant subtilases is a large insertion of about 120 amino acids within the catalytic domain, between the His and Ser residues of the catalytic triad. This protease-associated (PA) domain has been implicated in protein/protein interactions and substrate recognition in other proteins (26, 27), but its function in plant subtilases is unknown. We therefore embarked on a detailed biochemical characterization of a typical plant subtilase from tomato, SISBT3 (Solanum lycopersicum subtilase 3), which we obtained in high yields from a homologous expression system.

EXPERIMENTAL PROCEDURES

Overexpression Construct for SISBT3—The open reading frame of SISBT3 (7) (accession number AJ006376) was amplified by PCR using Pfu DNA polymerase (Stratagene, La Jolla, CA). Cloning into the movable expression cassette of pDH51 (28) was facilitated by SmaI and PstI restriction sites included in the PCR primers (all primer sequences are given in the supplemental material). The entire expression cassette for SISBT3 under control of the 35 S promoter and terminator sequences of the cauliflower mosaic virus was then cloned into the NdeI site of pSHI913, a derivative of pDH51 containing a kanamycin resistance gene to allow for selection of transformed plant cells (29).

Generation of a Cell Line for Overexpression of SISBT3—A cell suspension culture of wild tomato (Solanum peruvianum), maintained in a Murashige-Skoog type medium containing 5.0 mg/liter 1-naphthylacetic acid (30), was transformed by particle infiltration essentially as described (31), with some modifications. Briefly, a 50-ml culture was allowed to sediment 6 days after inoculation, and the culture supernatant was replaced with the same volume of fresh medium supplemented with 10% sucrose. After 30 min of gentle agitation, 2 ml of packed cell volume were distributed onto 0.6% agar plates prepared with the same high osmotic strength medium, and the SISBT3 overexpression construct was delivered into the cells by particle infiltration. For this purpose, the linearized construct was absorbed onto spherical gold particles (1.5–3 μm; Aldrich) in a calcium-spermidine precipitation procedure (31). By use of a particle inflow gun (Bio-Rad), 10 μg of the loaded microcarrier were shot into the cells under partial vacuum (0.8 bars). The cells were allowed to recover for 1 day on the transformation plates and for 1 week in 6 ml of culture medium on an orbital shaker (100 rpm; 25 °C). Transformed clones were then selected on culture plates supplemented with 75 μg/ml kanamycin. When initial callus growth was observed after 2–3 weeks, individual calli were isolated and maintained on the same selection medium. A cell suspension culture was established from the line showing the highest level of expression for SISBT3 (line I1I23) and maintained as described for the original S. peruvianum wild-type culture.

processed by AtS1P as well (12). The second ortholog of a known eukaryotic subtilase is AtSBT6.2, which was identified as tripeptidyl peptidase II, an intermediate exopeptidase necessary for efficient protein turnover and amino acid recycling (13).

The remaining 54 subtilases in Arabidopsis, all within the pyrolysin subfamily and absent from animals, may have adopted plant-specific functions during the course of evolution. This has, in fact, been confirmed for some of them, including AtSBT1.7, which is located in the seed coat, where it is supposed to modulate the activity of cell wall-modifying enzymes (14). AtSBT1.2 was shown to be involved in stomata development. Disruption of the AtSBT1.2 gene in the sdd1 ( stomatal density and distribution 1) mutant results in the clustering and increased density of stomates. This subtilase is secreted into the cell wall of meristemoids and guard mother cells, where it is thought to act as a processing protease in the generation of a signal responsible for stomata density regulation (15). The AtSBT2.4 gene is affected in the ale1 (abnormal leaf shape 1) mutant and may be involved in the generation of a peptide signal required for cuticle formation and epidermal differentiation during embryo development in Arabidopsis (16). However, the proposed function in the formation of a peptide signal has thus far been demonstrated for only a single subtilase; AtSBT1.1 was implicated in shoot regeneration, where it is required for the processing of pro-AtPSK4 (17), one of the six precursor proteins for phytosulfokines (i.e. mitogenic peptide growth factors in plants) (18).

With the physiological functions of plant subtilases beginning to emerge, there is an increasing need for a better understanding of these enzymes also at the biochemical and structural levels. As predicted from primary structure, most plant subtilases are secretory enzymes, which, like mammalian PCs, are synthesized as preproteins and targeted to the ER by virtue of their N-terminal signal peptides. In animals, much work has focused on the subsequent processing of thezymogens in the ER and its relevance for enzyme maturation and subcellular sorting. In a first intramolecular autocatalytic processing step, the prodomain is cleaved at its junction with the catalytic domain. This cleavage event is necessary for prodomain-assisted folding of the enzyme and exit from the ER. The prodomain remains covalently bound and acts as a specific inhibitor of proteolytic activity. Cleavage of the prodomain at a second site is required for inhibitor release and enzyme activation (see Refs. 19–21 and references therein). C-terminal extensions of the catalytic domain are similarly important for enzyme maturation and sorting. In kexin-like PCs the catalytic domain is joined to an eight-stranded β-sandwich, the P-domain, which is essential for folding and activity (22, 23). In the case of the membrane-bound PCs, the C terminus comprises a transmembrane domain and a cytosolic tail important for the subcellular distribution of the protease. Secreted PCs may remain tethered to the cell surface via a specific C-terminal cysteine-rich domain. For other PCs, the sorting into the regulated secretory pathway relies on specific signals also within their C-terminal regions (see Refs. 23–25 and references therein).
Generation of an Antiserum against SISBT3—The 1284-base pair EcoRI/MunI fragment of the cDNA coding for amino acids 207–636 of SISBT3 was expressed as a glutathione S-transferase fusion protein in *Escherichia coli*. The fusion protein accumulated intracellularly as insoluble inclusion bodies, which were isolated from cell extracts. After separation by SDS-PAGE, the gel slice containing the glutathione S-transferase-SISBT3 fusion protein was homogenized and used to immunize a rabbit (100 and 50 μg of protein in the first and three consecutive injections, respectively; Eurogentec, Seraing, Belgium). Serum was sampled 2 weeks after the fourth injection. A 1:2000 dilution was used on Western blots in combination with goat anti-rabbit IgG/horseradish peroxidase conjugate (Bio-Rad; diluted 1:10,000). The blots were developed by ECL (GE Healthcare).

Purification of SISBT3—The transgenic cell suspension culture expressing SISBT3 was harvested 8 days after inoculation, and the cells were separated from the medium by filtration. The filtrate was chilled to 4 °C and subjected to fractionated ammonium sulfate precipitation. The precipitate (60–85% saturation) from 6 liters of culture supernatant was resuspended in 60 ml of buffer A (25 mM Na2HPO4/NaH2PO4, pH 7.0; 5 mM EDTA), centrifuged to remove insoluble polysaccharides and cellular debris (2600 x g, 15 min, 4 °C), and dialyzed against buffer A. Proteins were then absorbed to a cation exchange matrix (SP-Sepharose FF; GE Healthcare) equilibrated in buffer A in a batch procedure. After washing in buffer A, proteins were eluted from the matrix with 1 M NaCl in buffer A and dialyzed twice against 4 liters of buffer B (25 mM Tris/HCl, pH 9.2, 5 mM EDTA). The dialysate was cleared by filtration (0.45 μm of cellulose acetate; Millipore, Schwalbach, Germany) and concentrated by ultrafiltration (Vivaspin, 10 kDa molecular mass cutoff; Vivascience, Hannover, Germany). The protein sample was subjected to anion exchange chromatography on Resource Q (6-ml column on an ÄKTA purifier chromatography system; GE Healthcare) equilibrated in buffer B. Protein was eluted with a linear gradient of NaCl in buffer B (0–1 M in 120 ml) at a flow rate of 3 ml/min. Fractions containing SISBT3 were pooled, concentrated by ultrafiltration, and stored in small aliquots at −80 °C. From 6 liters of cell culture supernatant, the procedure yielded 1.2 mg of pure SISBT3, as judged by SDS-PAGE.

Continuous Assay for SISBT3 Activity—A spectrofluorimetric assay was used to follow the enzymatic activity of SISBT3 during the purification procedure and for steady state kinetic analyses. In a total volume of 200 μl, the standard assay contained 10 μM aminobenzoyl-SKRDPKMQTD(NO2)Y (JPT Peptide Technologies, Berlin, Germany) and 4.8 μg of purified SISBT3 in 50 mM Tris/HCl, pH 8.0. Cleavage of the internally quenched substrate peptide was monitored over 30 min in a Cary Eclipse spectrofluorimeter (Varian, Darmstadt, Germany) (λex, 320 nm; λem, 420 nm). Kinetic constants were derived from three independent experiments, each involving seven substrate concentrations in the range from 2.5 to 200 μM. The data were fitted to the Michaelis-Menten equation by a nonlinear least squares method using the enzyme kinetics module in the Sigmaplot software (Systat Software GmbH, Erkrath, Germany).

MALDI-TOF Mass Spectrometry Assay—The hydrolysis of synthetic peptide substrates (glucagon, substance P, bradykinin, melittin, and the oxidized insulin B chain obtained from Sigma and systemin from Enzyme Systems Products (Livermore, CA)) by SISBT3 was analyzed by MALDI-TOF MS. The reaction was performed at room temperature in a total volume of 50 μl containing 50 mM Tris/HCl, pH 7.5, 1–2 pmol of SISBT3, and 2.5 nmol of peptide substrate. At various time points, 0.8-μl aliquots were taken, and the cleavage products were analyzed as described (32).

Protease Spot Assay—A collection of 72 fluorogenic peptides derived from the systemin sequence was synthesized by SPOT Technology (JPT Peptide Technologies). The peptides comprised an aminobenzoyl group as the fluorescent moiety at their N termini and were attached via their C termini to cellulose membranes in a 96-well microtiter plate. The assay was initiated by adding 200 μl of the assay mixture containing SISBT3 (24 ng/μl) in 50 mM Tris/HCl, pH 8.0, into each of the wells. The fluorescence (λex, 320 nm; λem, 420 nm) released from the membranes was analyzed in 50-μl aliquots taken after 0, 6, 24, and 30 h of incubation.

Characterization of SISBT3 and Identification of N-Glycosylation Sites by Mass Spectrometry—Detailed procedures are given in the supplemental material.

Active Site Mutants of SISBT3—Site-directed mutagenesis of the SISBT3 cDNA in the vector pSPT18 (Roche Applied Science) was done by PCR using complementary oligonucleotide primers incorporating the desired mutations. The mutagenic primers were extended during temperature cycling (95 °C, 30 s; 58 °C, 60 s, 72 °C, 7 min; 15 cycles) by Pwo DNA polymerase (Pq/Lab, Erlangen, Germany). Reaction products were digested at 37 °C overnight with 1 unit of DpnI (Fermentas, St. Leon-Rot, Germany) and transformed into XL1-blue (Stratagene)-competent bacteria. After sequence analysis to confirm the presence of the desired mutation, the mutated SISBT3 cDNAs were excised with Smal and PstI, blunt-ended with T4 DNA polymerase, and cloned into the Smal site of pART7 (33). The entire expression cassette containing the 35 S promoter, the mutated cDNA, and the nos terminator was then cloned into the binary vector pART27 (33) using the NotI restriction site. For transient expression in *Nicotiana benthamiana*, the vector was transformed into *A. tumefaciens* strain C58C1.

The c-Myc-tagged variants of SISBT3 and the S538A mutant were generated by insertion of a single c-Myc tag (EQKLISEEDL) five amino acids downstream of the cleavage site of the prodomain (between the prodomain and the catalytic domain, after Ser117). Briefly, a 618-bp EcoRI fragment from the 5′-end of the SISBT3 cDNA spanning the prodomain/catalytic domain junction was replaced by two PCR-generated cDNA fragments containing the tag. The PCR products were digested with EcoRI and ligated with either the wild-type SISBT3 cDNA or the S538A mutant in pART7 previously cut with EcoRI to release the 618-bp fragment from the 5′-end. The entire expression cassette was then cloned into the binary vector pART27. c-Myc-tagged proteins were detected on Western blots using the monoclonal mouse antibody 9E10 (hybridoma supernatant, 1:50; ATCC number CRL-1729) as the primary and anti-mouse.
IgG peroxidase conjugate (1:5000) as the secondary antibody, respectively.

**PA Domain Deletion Mutant**—To construct the PA domain deletion mutant lacking amino acids 363–456 of SISBT3, two fragments of the SISBT3 cDNA were synthesized by PCR using *Pwo* polymerase. The N-terminal fragment (amino acids 1–362) and the C-terminal fragment (amino acids 457–761) were digested with Smal and CiaI, respectively, and cloned jointly into the corresponding restriction sites of pART7. The entire expressing cassette was subsequently transferred into pART27.

**C-terminally Deleted SISBT3 Mutants**—C-terminal deletion mutants ending at codons 598, 705, 723, 742, 756, and 757 were constructed by PCR with a forward primer, which binds upstream of the XbaI site in the SISBT3 cDNA, and one of a series of reverse primers, each comprising a XbaI restriction site and a stop codon to terminate translation at the desired position. The SISBT3 fragments were amplified by PCR using *Pwo* polymerase, and the reaction products were cloned into pCR2.1 TOP (Invitrogen). The fragments were then mobilized from pCR2.1 TOP with XbaI and replaced the 870-bp 3′-end of the SISBT3 cDNA in pART7. The entire expression cassette was then moved from pART7 into pART27 and transformed into *A. tumefaciens* C58C1 as described above.

**Transient Expression in N. benthamiana**—Proteins were transiently expressed in *N. benthamiana* by agroinfiltration following published protocols (34) with minor modifications. The procedure involves co-infiltration of two *Agrobacterium* strains: *A. tumefaciens* C58C1 harboring the helper plasmid pCH32 (35) and one of the pART27 expression plasmids and *A. tumefaciens* C58C1 containing the p19 silencing plasmid (34). Both strains were cultivated for 20 h at 28 °C in YEB medium supplemented with the appropriate antibiotics. The cultures were harvested by centrifugation (2300 × g, 5 min) and resuspended in infiltration buffer (10 mM MES, pH 5.6, 10 mM MgCl₂). The two cultures were mixed to an A₅₉₀ of 0.7 and 1.0 for the expression plasmid and p19, respectively. Acetosyringone (150 μM) was added, and after 3 h of incubation, the bacterial cells were infiltrated into leaves of 6-week-old plants. The leaves were harvested after 5 days for protein extraction and analysis.

**Protein Isolation from N. benthamiana Leaves**—For total protein extracts, leaf material (1 cm²) was ground in liquid nitrogen and thawed in 250 μl of extraction buffer (50 mM Tris/HCl, pH 7.5, 100 mM NaCl, 0.5% Triton X-100, 10 mM β-mercaptoethanol). The samples were cleared by centrifugation, and the supernatant was used for further analysis or stored at −20 °C. Apoplastic proteins were isolated as described (36). Leaves were infiltrated with 300 mM NaCl in 50 mM sodium phosphate buffer, pH 7.0, and apoplastic washes were recovered by centrifugation for 7 min at 2000 × g and 4 °C.

**Glycosylation Assays**—Periodic acid–Schiff’s reagent was used to detect glycosylated proteins. Following SDS-PAGE, proteins were first fixed in the gel with several changes of 40% ethanol, 7% glacial acetic acid. Sugar residues were then oxidized for 1 h using 1% (w/v) NaIO₄ in 1% acetic acid, and gels were washed and stained by incubation in Schiff’s reagent with RNase B as a positive control. Endoglycosidase H (Endo H) and PNGase F were used according to the manufacturer’s instructions (New England Biolabs, Frankfurt, Germany). In a total volume of 30 μl, 20 μg of total leaf protein, 7.5 μg of apoplastic proteins, or 200 ng of purified SISBT3 were incubated for 2 h with 1500 units of Endo H and 1000 units of PNGase F, respectively. The reactions were terminated by the addition of 10 μl of 4× SDS sample buffer and analyzed by SDS-PAGE and immunoblotting using the polyclonal anti-SISBT3 serum.

**RESULTS**

**Overexpression and Purification of SISBT3**—The SISBT3 cDNA encodes a preproprotein (Fig. 1A), which may rely on the secretory pathway of a eukaryotic host cell for correct folding, processing, and activity. We therefore decided to use plant cells as a homologous overexpression system. The open reading frame of the SISBT3 cDNA was put under the control of the
strong and constitutive CaMV 35 S promoter and was stably introduced into cultured cells of wild tomato (S. peruvianum). Transgenic cell lines were analyzed for SlSBT3 expression on Western blots. From the line showing the highest expression level, a cell suspension culture was established that was found to accumulate SlSBT3 in the medium, with a maximum at 8 days after subculture (data not shown). SlSBT3 was purified from the supernatant of 8-day-old cultures by fractionated ammonium sulfate precipitation, batch adsorption to cation exchange material, and anion exchange chromatography.

This purification procedure proved highly efficient. One liter of cell culture yielded 200 μg of homogenous protein, which was identified as SlSBT3 by a polyclonal antiserum on Western blots (Fig. 2A). However, because of many related subtilases in tomato, additional proof was required to confirm the identity of SlSBT3. N-terminal sequence analysis resulted in a sequence of 113 amino acids (Fig. S1), which corresponds to amino acids 113–142, as predicted from the SlSBT3 cDNA, indicating that the prodomain was cleaved between His112 and Thr113 and identifying Thr113 as the N terminus of the mature protein. The closest homolog of SlSBT3 in tomato is SISBT4B (7), which deviates from the N-terminal sequence obtained here in position 28 (Ile instead of Val). Further confirmation for the identity of SISBT3 was provided by mass spectrometry. The peptides identified by liquid chromatography-electrospray ionization-tandem MS analysis of a tryptic digest of the purified protein cover 58% of the predicted SlSBT3 sequence (Fig. 1B).

The molecular mass of purified SISBT3, as determined by SDS-PAGE (83 kDa; Fig. 2A) and MALDI-TOF MS (79 kDa; Fig. S1) was found to be considerably higher than 69.5 kDa, as calculated from the protein sequence. Consistent with the observed mass difference, SISBT3 was identified as a glycoprotein by periodic acid-Schiff staining (Fig. 2B). The consensus motif for N-linked glycosylation (NX(S/T), with X being any amino acid except proline) occurs nine times in mature SISBT3. For seven of the potential glycosylation sites, the additional mass of attached sugar residues may explain why the corresponding tryptic peptides escaped detection during liquid chromatography-electrospray ionization-tandem MS analysis (Fig. 1B). Manual inspection of the mass spectra did indeed reveal the presence of glycosylated peptides but did not allow for identification of the glycosylation sites because the spectra were dominated by glycan fragment ions (data not shown). We therefore decided to enrich glycopeptides by hydrophilic interaction chromatography and subsequent MALDI-TOF MS analysis (Fig. S2). Enriched glycopeptides were fragmented by MALDI-TOF/TOF, leading to the identification of both complex and paucimannosidic type glycan chains at Asn177, Asn203, Asn376, Asn409, Asn627, Asn644, and Asn666 could not be identified at all; therefore, additional glycosylation at these three sites cannot be excluded.

Catalytic Properties of SISBT3—The proteolytic activity of SISBT3 was analyzed using an internally quenched fluorogenic peptide (aminobenzoyl-SKRDPKMQTDLY(NO2)) as the substrate. Its sequence was derived from the peptide systemin, which had been identified as an SlSBT3 substrate during the initial characterization of substrate specificity (see below). The pH dependence of SISBT3 activity was determined in the range from pH 4 to 11 in a tricomponent buffer system of constant ionic strength (37). As shown in Fig. 3A, SISBT3 displayed highest activity under alkaline conditions, with a maximum between pH 7.5 and 8.0, and 60% of the activity was retained at pH 11.0.

For the analysis of thermostability, the enzyme was heat-treated for 20 min and chilled on ice, and the residual proteolytic activity was assayed at 25 °C. SISBT3 activity was unaffected at 60 °C and only partially reduced after incubation at 70 °C (Fig. 3B). SISBT3 thus resembles other plant subtilases (38–40), exhibiting a considerable degree of thermostability. Consistent with its identity as a serine proteinase, SISBT3 activity was fully inhibited by 1 mM 4-(2-aminoethyl)-benzenesulfonyl fluoride, a general inhibitor of serine proteinases, but not by the more selective inhibitors TLCK, TPCK, leupeptin, aprotinin, or benzamidine (Fig. 3C). Inhibitors of cysteine proteases (N-trans-epoxysuccinyl-l-leucine-4-guanidinobutyralamide; 50 μM) and metalloproteases (EDTA and EGTA; 5 mM), bivalent cations (Ca2+ and Mg2+; 5 mM), the reducing agent 1,4-dithio-
Enzymatic properties of SlSBT3.

Each experiment was performed in triplicate, using three independently purified batches of recombinant A, pH optimum. SlSBT3 activity was assayed at different pH values in a tricomponent buffer system of constant ionic strength. B, heat stability. The assays were incubated for 20 min at the temperatures indicated and subsequently chilled on ice. Each reaction was started by the addition of substrate, and residual SlSBT3 activity was assayed at 25 °C. Substrate specificity of SlSBT3 — In order to obtain a first impression of the substrate preference of SlSBT3, synthetic oligopeptide substrates (systemin, glucagon, substance P, bradykinin, melittin, and oxidized insulin B-chain) were incubated with the purified enzyme, and the cleavage products were analyzed by MALDI-TOF MS. A compilation and ranking of the observed cleavage sites are shown in Fig. 4. Any site that was fully processed after 2 h was considered a better substrate for SlSBT3 as compared with sites that were only partially cleaved even after 15 h. A comparison of the various cleavage sites revealed a preference of recombinant SBT3 for Gln in the P1 position of its substrates (nomenclature according to Schechter and Berger (41)). However, this preference was not absolute, since some degree of processing was also observed at the carboxyl side of Val, Ser, Asp, His, Pro, Ala, and Leu (Fig. 4).

The 18-amino acid peptide systemin turned out to be the best substrate and was cleaved at a single site (i.e. carboxyl-terminal of Gln16). In order to analyze the contribution of the individual amino acids to substrate recognition, systemin analogs with alanine substitutions at each position along the sequence were tested as SlSBT3 substrates in the mass spectrometry assay. All 17 Ala-substituted systemin derivatives were cleaved by SlSBT3 except the Q16A-analogue, indicating that Gln16 is the only amino acid within the systemin sequence that is essential for substrate recognition (data not shown).

For further refinement of SISBT3 substrate specificity, the Protease Spot assay (JPT Peptide Technologies) was used, employing 72 fluorogenic peptides bound to cellulose discs. These peptide substrates were derived from the systemin sequence and were substituted between the positions P3 and P2' with Ala, Phe, Pro, Ser, Asn, Asp, Lys, Val, Leu, or His (to represent aliphatic versus aromatic, basic versus acidic, and polar versus unpolar residues). In this semiquantitative assay, four peptides were identified that were cleaved at least twice as fast as compared with systemin, including the peptides with His substituted for Gln in the P1 position and with Lys substitutions in positions P3, P2', and P1'. The results indicate a preference of SlSBT3 for basic substrates with Gln or His on the amino side of the scissile bond. To substantiate this hypothesis, a series of alanine substitutions at each position along the sequence were fitted to the Michaelis-Menten equation.

Biochemical Characterization of SBT3 in Tomato

**Figure 3. Enzymatic properties of SISBT3.** Each experiment was performed in triplicate, using three independently purified batches of recombinant SISBT3. A, pH optimum. SISBT3 activity was assayed at different pH values in a tricomponent buffer system of constant ionic strength. B, heat stability. The assays were incubated for 20 min at the temperatures indicated and subsequently chilled on ice. Each reaction was started by the addition of substrate, and residual SISBT3 activity was assayed at 25 °C. C, inhibitor profile. SISBT3 was incubated for 20 min in assay buffer containing the different protease inhibitors. Inhibitor concentration is given in brackets in mM. Reactions were started by the addition of substrate. In A–C, activity is expressed as a percentage of the highest value or control, with 100% corresponding to 111 ± 13 pmol/min, 151 ± 36 pmol/min, and 143 ± 16 pmol/min in A, B, and C, respectively. D, steady state kinetic analysis. In three independent experiments, SISBT3 activity (nmol/min) was assayed at substrate concentrations ranging from 2.5 to 200 μM. In order to derive the kinetic constants, the data were fitted to the Michaelis-Menten equation.
Biochemical Characterization of SBT3 in Tomato

TABLE 1
Kinetic analysis of substrate specificity

| Substrate | P5 | P4 | P3 | P2 | P1 | P1' | Kₚₑₗ ± S.E. | Vₚₑₗ ± S.E. | kₚₑₗ/Kₚₑₗ |
|-----------|----|----|----|----|----|-----|----------------|---------------|-------------|
| Systemin: | P  | P  | K  | M  | Q  | T   | 32.8 ± 4.6     | 0.151 ± 0.007 | 1.29        |
| Pep1      | P  | D  | K  | K  | Q  | T   | 30.7 ± 6.6     | 0.888 ± 0.072 | 8.00        |
| Pep2      | P  | D  | K  | K  | M  | H   | 66.7 ± 18.1    | 0.011 ± 0.020 | 0.05        |
| Pep3      | P  | D  | K  | K  | H  | T   | 53.5 ± 7.0     | 0.143 ± 0.007 | 0.74        |
| Pep4      | P  | D  | K  | K  | H  | K   | 40.2 ± 6.0     | 0.131 ± 0.007 | 0.90        |

| Systemin: | AVQSKPPSKRDPKKMTD |
| Glucagon: | HQGTFSDKYSYLDSSRAQDFVQLMNT |
| Substance P: | RRPKKQFFGLM |
| Insulin: | FVQHLC(SO3H)GSHLEAYLVC(SO3H)GERGFFTYPKA |
| Mellitin: | GIGAVKLVTTLGPALISWIKRRQQ |
| Neurotensin: | PeLYENKPRRPYIL |

FIGURE 4. Cleavage of synthetic peptides by SBT3. Synthetic peptides were incubated with recombinant SBT3, and the cleavage products were analyzed by MALDI-TOF mass spectrometry after 2 and 15 h, respectively. Sites that were efficiently processed after 2 h are indicated by heavy arrows; lighter arrows mark sites that were only partially processed even after 15 h of incubation. Gln residues in the P₁ position of cleaved sites are underlined.

internally quenched fluorogenic substrates was synthesized and analyzed kinetically. For all of the substrates tested, there were only small differences in Kₚₑₗ, and the observed changes in catalytic efficiency resulted to a large degree from different reaction rates. The results are summarized in Table 1 and confirm that His is tolerated in the P₁ position of SBT3 substrates. However, the catalytic efficiency for this peptide was found to be almost 30-fold reduced as compared with systemin. Consistent with the results from the protease spot assay, Lys substitutions resulted in a substantial increase in the rate of reaction and, consequently, higher catalytic efficiency. The introduction of Lys in P₂ caused 6.2- and 14.8-fold increases in kₚₑₗ/Kₚₑₗ for the peptide substrates with Gln and His in P₁, respectively (compare Pep₁ versus systemin and Pep₃ versus Pep₂; Table 1). A further small increase resulted from an additional Lys substitution in P₁' (compare Pep₃ and Pep₄; Table 1). The data indicate that basic peptides with Gln and Lys in the P₁ and P₂ positions are preferred substrates of SBT3. Consistent with the observed substrate specificity, SBT3 was not inhibited by TLCK or TPCK, which target serine proteinases with basic or large hydrophobic residues in P₁ (trypsin and chymotrypsin-like activities, respectively; Fig. 2C).

Prodomain Processing and Secretion of SBT3—Processing of the prodomain is known to occur autocatalytically in bacterial, yeast, and animal subtilases (42–44). In most mammalian PCs, the junction between the prodomain and the catalytic region is cleaved in a first intramolecular reaction in the ER, and this cleavage is necessary for the protein to pass ER quality control and for continued sorting within the secretory pathway. Only PC2 exits the ER as azymogen, and cleavage of the prodomain occurs late in the secretory pathway (45). We decided to address these questions for plant subtilases and investigated first whether prodomain processing in SBT3 is an autocatalytic event.

Active site mutants of SBT3 were generated in which the Ser residue of the catalytic triad (Ser₅₃₈) was replaced by alanine or cysteine. The processing and targeting of the S538A and S538C mutants were compared with the wild type enzyme in a transient expression system (agroinfiltration of N. benthamiana leaves). For wild-type SBT3, the processed ~80-kDa mature enzyme was the predominant form, and the ~100-kDa precursor could only be detected as a minor band on Western blots of total protein extracts (Fig. 5A, top). In contrast, only the precursor protein was detectable for the two active site mutants, suggesting that SBT3 activity is required forzymogen maturation and that precursor processing is autocatalytic. In the next step, we used a Myc-tagged version of the S538A mutant in order to investigate whether the precursor is processed in an intra- or in an intermolecular reaction. In contrast to Myc-tagged wild-type SBT3, which was processed and secreted and unaffected in its mobility during SDS-PAGE (data not shown), S538A-myc was detected as the larger unprocessed precursor could only be detected as a minor band on Western blots of total protein extracts (Fig. 5A, top). In contrast, only the precursor protein was detectable for the two active site mutants, suggesting that SBT3 activity is required forzymogen maturation and that precursor processing is autocatalytic. In the next step, we used a Myc-tagged version of the S538A mutant in order to investigate whether the precursor is processed in an intra- or in an intermolecular reaction. In contrast to Myc-tagged wild-type SBT3, which was processed and secreted and unaffected in its mobility during SDS-PAGE (data not shown), S538A-myc was detected as the larger unprocessed precursor could only be detected as a minor band on Western blots of total protein extracts (Fig. 5A, top). In contrast, only the precursor protein was detectable for the two active site mutants, suggesting that SBT3 activity is required forzymogen maturation and that precursor processing is autocatalytic. In the next step, we used a Myc-tagged version of the S538A mutant in order to investigate whether the precursor is processed in an intra- or in an intermolecular reaction. In contrast to Myc-tagged wild-type SBT3, which was processed and secreted and unaffected in its mobility during SDS-PAGE (data not shown), S538A-myc was detected as the larger unprocessed precursor could only be detected as a minor band on Western blots of total protein extracts (Fig. 5A, top). In contrast, only the precursor protein was detectable for the two active site mutants, suggesting that SBT3 activity is required forzymogen maturation and that precursor processing is autocatalytic.
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F-resistant, which is consistent with the presence of N-linked fucosylated glycans, as detected by mass spectrometry in mature and secreted SISBT3. In contrast, the S538A and S538C mutant proteins were sensitive to PNGase F digestion, confirming that they had not yet reached the medial Golgi and trans-Golgi (Fig. 5C). To exclude the possibility that the observed shift in electrophoretic mobility is due to protease contaminations in the Endo H and PNGase F preparations, glycosidase treatments were repeated in the presence of a mixture of protease inhibitors, and identical results were obtained (compare Fig. 5C and supplemental Fig. S4).

Processing and Secretion of C-terminal Mutants—To assess the relevance of the C terminus for processing and secretion, a series of C-terminally truncated SISBT3 mutants was constructed (Fig. 6A) and transiently expressed in N. benthamiana. Fig. 6B shows that all mutants lacking more than five amino acids from their C termini (i.e. those shorter than SBT-756) were expressed as unprocessed precursors that failed to be secreted. For SBT-756, the processed band was just barely detectable, whereas partial processing and secretion were observed for SBT-757. All longer mutants (i.e. SBT-758 to SBT-761) were fully processed as well as secreted. The two processes, prodomain processing and secretion, appear to be linked, which is in agreement with the notion that subtilase activity and autocatalytic cleavage of the prodomain are prerequisites for passage through the secretory pathway.

DISCUSSION

The large family of pyrolysin-related subtilases in plants comprises both nonspecific degradative proteinases and highly specific processing enzymes (9, 48). Examples of degradative subtilases include cucumisin, which constitutes up to 10% of the soluble protein in melon fruit and cleaves a broad variety of peptide and protein substrates (49, 50), and maclurilisin from the fruits of Maclura pomifera, for which similar characteristics have been reported (51). For many of the selective plant subtilases, on the other hand, there is only genetic evidence for their specific regulatory functions, and very little is known about the properties of the enzymes (14, 16, 17, 52). These subtilases are typically expressed at low levels in specific cell types or tissues or during a limited time in plant development, which necessitates suitable overexpression systems to allow for their purification and characterization.

We developed a transgenic plant cell suspension culture as a homologous overexpression system, yielding 200 μg of homogenous SISBT3 protein/liter of cell culture. This is somewhat less efficient as compared with the baculovirus/insect cell system that was previously used to express the subtilase SBT1 from tomato at 1.5 mg/liter (53). However, taking advantage of the homologous expression system, we were able to identify the native glycosylation pattern and path of maturation for SISBT3.

SISBT3 was found to be glycosylated at five of nine potential N-linked glycosylation sites (Asn↑177, Asn↑203, Asn↑376, Asn↑541, and Asn↑545; Fig. S3). Considerable heterogeneity was observed with respect to oligosaccharide structures, including both paucimannosidic type glycans and complex glycans processed to varying degrees (supplemental Table S1). Complex type N-glycans are synthesized during passage from the cis-Golgi
A range of C-terminally deleted SlSBT3 mutants was generated, which are shown schematically in C-terminal requirements for processing and targeting of FIGURE 6.

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through medial Golgi to trans-Golgi cisternae involving the trimming of high mannose precursors and the addition of N-acetylglucosamine to the two terminal mannoses, followed by the addition of fucose and galactose residues. Additional plant-specific modifications include α(1,3)-fucosylation and β(1,2)-xylosylation of the glycan core (47). After maturation in the Golgi, complex N-glycans continue to be modified en route to their final destination. Although unmodified complex type glycans are typically found in extracellular proteins, extensive trimming has been observed for vacuolar glycoproteins, resulting in the formation of paucimannosidic type glycans, which retain just the fucosylated and xylosylated pentasaccharide core structure (Man$_2$-Xyl-GlcNAc$_2$-Fuc) (47). The prevalence of this type of modification in SISBT3 thus appears exceptional. There is precedence, however, for paucimannosidic glycans also in other extracellular proteins, and the enzymes presumably involved in the processing were characterized recently (see Refs. 54 and 55 and references therein). Complex type glycans truncated to varying degrees were observed at four of the five glycosylation sites, and the largest glycan structure was retained at Asn$^{745}$ (Table S1). Limited processing at this site is likely to reflect the limited accessibility of the oligosaccharide substrate to processing enzymes (47).

Three of the glycosylation sites in SISBT3 (Asn$^{177}$, Asn$^{697}$, and Asn$^{745}$) were not recognized by NetNGlyc, a World Wide Web-based prediction server for N-linked glycosylation in the NXS/T sequence context of human proteins (56). Asn$^{127}$, on the other hand, was predicted to be glycosylated by NetNGlyc but was found to be sugar-free in SISBT3. Human glycoproteins, on which NetNGlyc had been trained, are glycosylated most frequently within their N-terminal regions (one- to two-fifths into the protein chain from the N terminus), whereas the C terminus is clearly disfavored for glycosylation (56, 57). In contrast, the two most C-terminal NX(S/T) sequons were found to be glycosylated in SISBT3 (Asn$^{697}$ and Asn$^{745}$). These findings point to differences between plants and mammals with respect to the site specificity of N-linked glycosylation and emphasize the need for more experimental data to improve existing prediction tools for plant glycoproteins.

The determinants and subcellular site of maturation, on the other hand, appear to be similar between SISBT3 and the homologous mammalian PCs. We found that prodomain processing in SISBT3 is an intramolecular autocatalytic event. The inactive active site serine-to-alanine (S538A) mutant accumulated intracellularly as unprocessed zymogen, and processing in trans by wild-type SISBT3 was undetectable (Fig. 5). Also, the S538C mutant failed to be processed, indicating that the cysteine thiol cannot substitute for the serine hydroxyl as the nucleophile in the attack of the peptide bond at the carbonyl carbon (Fig. 5). In bacterial subtilisin E, on the other hand, the active site serine-to-cysteine mutant retained sufficient activity to allow prodomain processing but not the cleavage of other substrates (58). Similar to the active site mutants, any other mutation impairing prodomain processing (deletion of the PA domain (Fig. 5A) or serial deletions of the C terminus (Fig. 6)) affected not only zymogen maturation but also secretion. The two events could not be separated, suggesting that SISBT3 activity and prodomain processing are prerequisites for subcellular sorting along the secretory pathway.

Prodomain function and processing have been investigated in detail in kexin-like PCs and most intensively in furin. The prodomain of furin is cleaved at the junction to the catalytic domain in a rapid intramolecular reaction in the ER, and this cleavage is necessary for the protein to fold into its native state and for continued sorting along the secretory pathway (19, 44). As shown here for SISBT3, furin active site mutants fail to excise the prodomain, and they accumulate as unprocessed zymogens in the early secretory pathway (19, 59). The subcel-
lular sites of pro-furin accumulation have been identified as the ER-Golgi intermediate compartment and the cis-Golgi network (19). Endo H and PNGase F sensitivity observed here for the unprocessed SISBT3 mutants (Fig. 5C) is consistent with the findings for furin (and other mammalian PCs), suggesting that prodomain processing occurs in the ER as a prerequisite for secretion beyond the cis-Golgi.

For many of the mammalian PCs, C-terminal extensions to the catalytic domain have been shown to harbor additional determinants of subcellular localization. The cycling of furin, PC5B, and PC7 from the cell surface back to the trans-Golgi network, for example, is mediated by signals in their cytosolic tails (see Ref. 60 and references therein). Other examples include the RGD motif present in the C-terminal extensions of all PCs except PC7 and suggested to be required for sorting into the regulated secretory pathway (61, 62). Secretion of SISBT3 via the constitutive secretory pathway, on the other hand, is not expected to require additional sorting signals. Nevertheless, the C terminus of SISBT3 was found to be necessary for secretion. Terminally truncated mutants lacking five or more amino acids from the C terminus were retained intracellularly as unprocessed precursor proteins (Fig. 6). It is thus conceivable that targeting information is present within the primary structure of SISBT3 at about five amino acids from the C terminus, the conserved SPI motif (Fig. 6) being a candidate signal. More likely, however, the carboxyl-terminal domain is required for SISBT3 activity and, consequently, prodomain processing and secretion. This conclusion is supported by the observation that in serial deletion mutants, prodomain processing and secretion were inextricably tied events (Fig. 6).

Consistent with prodomain processing being autocatalytic, the amino acid sequence at the prodomain junction typically reflects the substrate specificity of the enzyme, at least in mammalian PCs. In furin, for example, prodomain processing occurs after its consensus cleavage site, Arg-Thr-Lys-Arg (19). Intriguingly, this is not the case in plant subtilases. A large insertion of 120 amino acids from the C terminus was retained intracellularly as unprocessed precursor proteins (Fig. 6). It is thus conceivable that targeting information is present within the primary structure of SISBT3 at about five amino acids from the C terminus, the conserved SPI motif (Fig. 6) being a candidate signal. More likely, however, the carboxyl-terminal domain is required for SISBT3 activity and, consequently, prodomain processing and secretion. This conclusion is supported by the observation that in serial deletion mutants, prodomain processing and secretion were inextricably tied events (Fig. 6).

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thought to mediate the interaction of the transmembrane and plant vacuolar sorting receptors with their respective ligands (26, 64), and the interaction of an E3 ubiquitin ligase (GRAIL) with its target (CD40L) (65). In a subtilase from Lactobacillus lactis, the PA domain was shown to affect substrate specificity (66) and was suggested to achieve this by binding the substrate and presenting cleavage sites to the active site of the protease (26). It is thus an interesting possibility that in plant subtilases, the PA domain may mediate autocatalytic processing by binding to the prodomain and presenting the prodomain junction to the active site as an intramolecular substrate. This intramolecular cleavage site appears to be characterized by a pair of threonine residues at the carboxyl side of the scissile bond rather than the variable amino acids on the amino side. An invariant pair of threonine residues was identified at the amino terminus of SISBT3 (Fig. 1) and other subtilases purified from plant tissues (39, 49, 51, 67). This sequence motif is in fact conserved in all subtilase sequences known from tomato (7, 68) and in all of those Arabidopsis subtilases that also possess a PA domain (54 of 56 sequences (9)). Although a role for the PA domain in prodomain processing remains hypothetical at this time, it is interesting to note that prodomain processing was indeed impaired in a PA domain deletion mutant of SISBT3 (Fig. 5A).

Alternatively, the PA domain may also support intermolecular substrate recognition and contribute to the binding of physiological substrates in vivo. The turnover numbers and catalytic efficiencies observed for SISBT3 with synthetic oligopeptide substrates were rather low ($k_{cat} = 0.25 s^{-1}$ for the best substrate peptide; Table 1) but similar to those reported for, for example, soybean C1 subtilase ($0.32–1.2 s^{-1}$ for different synthetic peptide substrates) (63). The low turnover numbers may result from suboptimal enzyme-substrate interaction, suggesting that the short oligopeptide substrates used to study subtilase activity in vitro may not comprise all of the features necessary for efficient substrate recognition in vivo. In fact, only peptides of at least 8 or 9 amino acids spanning positions P$_2$ to P$_4$’ (63) were cleaved efficiently by soybean C1 protease. Likewise, hordisin from barley requires four amino acids on either side of the scissile bond for efficient cleavage of peptide substrates. The catalytic efficiency with shorter peptides truncated by one or two amino acids on either side was reduced by 2–3 orders of magnitude (40). These observations suggest that the substrate binding cleft may be more extended in (at least some) plant subtilases as compared with the prototypical subtilisin BPN’ from Bacillus amyloliquefaciens, which makes contact with only six residues spanning P4–P2’ of the substrate (4). Alternatively, substrate binding may not be limited to the active site of the protease but may also include exosite interactions possibly involving the PA domain. In this way, the PA domain may contribute to the recognition and efficient cleavage of physiological substrates in vivo.

The identification of the physiological substrates and the in vivo function(s) of SISBT3 will be major tasks for the future. The efficient cleavage of systemin by SISBT3 in vitro and the central role of systemins as regulators of the wound response in plants of the nightshade family (including tomato) (69) point to a possible involvement of SISBT3 in the degradation of systemin in vivo and the modulation of the wound response. Transgenic
plants affected in elements of the wound response pathway and in the expression of SISBT3 will be used to test such a functional link between SISBT3 and the systemic signaling pathway.

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