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*Le*SBT1, a Subtilase from Tomato Plants

OVEREXPRESSION IN INSECT CELLS, PURIFICATION, AND CHARACTERIZATION*

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The cDNA of a tomato subtilase designated *Le*SBT1 was cloned from a tomato flower cDNA library. The deduced amino acid sequence indicated for *Le*SBT1 the structure of a prepro-protein targeted to the secretory pathway by virtue of an amino-terminal signal peptide. *Le*SBT1 was expressed in the baculovirus/insect cell system and a processed 73-kDa form of *Le*SBT1, lacking both signal peptide and prodomain, was purified to homogeneity from culture supernatants. This 73-kDa *Le*SBT1, however, lacked proteolytic activity. Zymogen activation to yield 68-kDa *Le*SBT1 required the additional processing of an amino-terminal autoinhibitory peptide in a strictly pH-dependent manner. Mature 68-kDa *Le*SBT1 showed highest activity at acidic pH consistent with its presumed localization in the apoplast of the plant cell. In comparison to other plant subtilases, *Le*SBT1 exhibited a narrower substrate specificity in that it cleaves only polypeptide substrates preferentially but not exclusively carboxyl-terminal of glutamine residues. The possible involvement of *Le*SBT1 in selective proprotein processing is discussed with reference to the related mammalian proprotein convertases.

Until recently, and in contrast to the (chymo)trypsin superfamily, serine proteases of the subtilisin superfamily (subtilases) appeared to be restricted to prokaryotes. The linear arrangement of the catalytic triad of Asp, His, and Ser residues differs between the primary structures of (chymo)trypsin and subtilisin-related proteases, and these proteases thus have served as a prime example of convergent evolution. More recently, however, subtilases have been discovered also in lower and higher eukaryotes, which further fueled the interest in this clan of serine proteases. Over 200 subtilases are presently known, and they have been grouped into six distinct families, three of which (the bacterial subtilisins, the eukaryotic kexins, and the pyrolysins of diverse origin) will be relevant for the following discussion (1).

The characterization of the yeast kex2 endoprotease involved in the processing of the α-factor pheromone and of killer toxin paved the way for the discovery of mammalian kexins or proprotein convertases (PCs).[1] Presently, seven PCs are known that characteristically cleave the polypeptide precursors of peptide hormones and neuropeptides carboxyl-terminal of paired basic residues (2, 3). In higher plants, there was initially only circumstantial evidence for the existence of kexin-like proteases. In tomato plants, a protein has been identified (SBP50) that specifically interacts with the polypeptide-wound signal molecule systemin. This protein was found to cross-react with an antiserum against a *Drosophila* PC (4). Furthermore, a kex2p-like pathway was shown to exist in tobacco by use of transgenic plants overexpressing the virally encoded antifungal KP6 killer toxin. Correct processing of the protoxin was observed, which required a kex2p-like activity within the secretory pathway of tobacco cells (5). The protease was later shown to be a Golgi-resident enzyme with a substrate specificity characteristic of yeast kex2p (6). The first subtilase to be cloned from a higher plant was cucumisin, an extracellular protease highly abundant in melon fruit (7). According to its primary structure, cucumisin was classified not as a kexin, but rather as a member of the pyrolysin family of subtilases (1). Unlike the kexins, cucumisin does not exhibit a preference for substrates with paired basic residues. Its substrate specificity was shown to be broad and similar to that of bacterial subtilisins (8, 9) and the plant proteases taraxalisin (10) and macluralisin (11). In recent years, genomic and cDNA sequences have been obtained for many plant subtilases from members of genera as diverse as *Lilium* (12) *Alnus* (13), *Arabidopsis* (13, 14), and *Lycopersicon* (15–18). The proteins derived from these sequences are all related to cucumisin and belong to the pyrolysin family of subtilases, which may suggest that plant subtilases (unlike the kexins) have a broad substrate specificity and serve degradative functions like bacterial subtilisins. The recent discovery of two mammalian pyrolysins indicates that this is not necessarily so. The Site-1 protease (S1P) and the subtilisin/kexin-isozyme-1 (SKI-1) exhibit exquisite substrate specificities that markedly differ from that of kexins. They have been implicated in the processing of sterol regulatory element-binding protein transcription factors and pro-brain-derived neurotrophic factor, respectively (19, 20).

We are interested in the function of subtilases in plant growth and development and in their possible role in proprotein processing. We have chosen tomato plants as the system to approach these questions, since most of the data on plant subtilases have been obtained in this species. At least 15 subtilases (SBTs) exist in tomato (*Lycopersicon esculentum*, *Le*), which can be grouped into five distinct subfamilies. Single genes were shown to exist for *Le*SBT1, *Le*SBT2, and tmp, while

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) X98929.

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[1] The abbreviations used are: PC, proprotein convertase; GST, glutathione *S*-transferase; MALDI-TOF, matrix-assisted laser desorption/ionization-time of flight; MES, 2-(*N*-morpholino)ethanesulfonic acid; PAGE, polyacrylamide gel electrophoresis; PCR, polymerase chain reaction; S1P, site-1 protease; SKI-1, subtilisin/kexin-isozyme-1; BisTris, bis(2-hydroxyethyl)iminotris(hydroxymethyl)methane; SBT, subtilase.

five and six genes were found in the *Le*SBT3/4 and P69 subfamilies, respectively (18). Thus, the complexity of the subtilisin family in tomato plants exceeds that of mammalian subtilases. While the tmp protease has been implicated in microspore development (12), two proteases of the P69 subfamily (P69B and P69C) are involved in the pathogen defense response (15–17). The function of *Le*SBT1, -2, -3/4, and the remaining P69 proteases is still unknown. With the notable exception of LRP, a tomato cell wall protein that is cleaved by one of the pathogen-inducible P69 proteases (21), the *in vivo* substrates of tomato subtilases have not been identified yet and, furthermore, none of these enzymes has been characterized biochemically. Clearly, biochemical data are needed to define the roles of these enzymes in plant growth and development. As a first step toward this objective, we describe in the present study the cloning of the *Le*SBT1 cDNA, the overexpression of the encoded protein in insect cell cultures, the purification of recombinant *Le*SBT1, and the characterization of its activity.

EXPERIMENTAL PROCEDURES

Cloning of the LeSBT1 cDNA—All basic molecular techniques were adapted from Sambrook *et al.* (22) or Ausubel *et al.* (23).

The PCR-generated probe used for the isolation of the *Le*SBT1 cDNA has been described and was used previously for the isolation of a genomic clone of *Le*SBT1 (18). Briefly, primers were designed corresponding to conserved regions between the catalytic domains of animal proprotein convertases and cucumisin (7) and were used in PCR reactions with tomato (*L. esculentum* Mill. cv. Castlemart II) genomic DNA as the template. Reaction products were cloned into Bluescript pSK− (Stratagene, La Jolla, CA). A PCR product exhibiting sequence similarity with subtilases was used for the screening of a tomato flower cDNA library in λZAPII (Stratagene) (24). A total of 4×10^5 phages were screened on duplicate nitrocellulose filters by hybridization to the radiolabeled cDNA probe (Prime-It system; Stratagene) as described (18). Three consecutive rounds of screening were performed for plaque purification of 16 positive λ phage clones. Recombinant pBluescript cDNA phagemids were excised *in vivo* using ExAssist helper phage according to the manufacturer's instructions (Stratagene). The clone with the longest cDNA insert designated *Le*SBT1 was chosen for further analysis. For sequence analysis, nested deletions were generated from the 5′end of the cDNA using exonuclease III and SI nuclease according to standard protocols (22). Reaction products were treated with the Klenow fragment of *Escherichia coli* DNA polymerase I, gel-purified, and cloned into pSK−. The coding strand of the cDNA inserts of the resulting plasmids were sequenced using fluorescent dideoxy chain terminators in the cycle sequencing reaction (Perkin-Elmer) and the Applied Biosystems model 373A DNA sequencer. Oligonucleotide primers were synthesized (Microsynth, Balgach, Switzerland) based on the obtained sequence information to facilitate the sequencing of the second strand of the *Le*SBT1 cDNA. Sequence data were compiled and analyzed using the University of Wisconsin GCG sequence analysis software package (25).

Northern Blot Analysis—Different organs of tomato plants were ground in liquid nitrogen, and the powder was subjected to RNA extraction using a phenol-based extraction procedure (22). Five μg of total RNA were separated on formaldehyde agarose gels, transferred to nitrocellulose membranes, and hybridized to the radiolabeled *Le*SBT1 cDNA using standard laboratory procedures (22). Prehybridization, hybridization, and washing of the filters was performed under conditions that did not allow cross-hybridization of the *Le*SBT1 probe with other members of the subtilase family in tomato (18).

Generation of a LeSBT1 Antiserum—The 870-base pair *Mun*I fragment of the *Le*SBT1 cDNA coding for amino acids 471–760 of the *Le*SBT1 protein was cloned into the *Eco*RI site of pGEX-2T (Amersham Pharmacia Biotech, Zürich, Switzerland) to generate pGEX-SBT1. pGEX-SBT1 was transformed into *E. coli* DH5, and its identity was confirmed by sequence analysis. A 100-ml culture was grown from a single transformant to a OD_{600} of 0.9, and expression of the *Le*SBT1 fragment in fusion with glutathione *S*-transferase (GST) was induced by addition of isopropyl-1-thio-β-D-galactopyranoside to a final concentration of 0.5 mM. After 3 h, cells were harvested by centrifugation. The GST-*Le*SBT1 fusion protein was found to be insoluble in *E. coli*, and it accumulated in inclusion bodies, which were purified from bacterial extracts as described (26). The fusion protein was further purified by SDS-PAGE (10% gels, buffer system as described by Laemmli (Ref. 27)) and the gel slice containing GST-*Le*SBT1 was used to raise a polyclonal antiserum (Eurogentec, Seraing, Belgium). Homogenized gel slices containing approximately 100 μg and 50 μg of fusion protein were used for the first and the three consecutive injections, respectively. Serum was sampled 2 weeks after the fourth injection. The serum was diluted 1:1000 to be used on Western blots in combination with a goat anti-rabbit IgG/alkaline phosphatase conjugate (Bio-Rad; diluted 1:3000).

Insect Cell Culture—Spodoptera frugiperda cells (IPLB-SF21, CLONTECH, Palo Alto, CA) were maintained in 15-ml monolayer cultures at 28 °C in Grace's insect medium (Life Technologies AG, Basel, Switzerland) supplemented with 10% fetal calf serum (Life Technologies AG), 50 units/ml streptomycin, and 50 units/ml penicillin. Cells (2×10^6) were transferred to fresh medium once a week. For overexpression experiments, *Sf*21 cells were maintained in 100-ml suspension cultures in serum-free medium (*Sf*-900 supplemented basal powdered medium, Life Technologies AG) containing 12.5 units/ml penicillin, 12.5 units/ml streptomycin, and 0.1% (w/v) Pluronic F68 at 28 °C at 140 rpm. Cells (5×10^7) were transferred to fresh medium once a week.

Generation of Recombinant Baculovirus—The open reading frame of the *Le*SBT1 cDNA was amplified by PCR using 1 unit of *Taq* DNA polymerase (Stoffel-fragment, Perkin-Elmer). Thirty cycles of amplification (95 °C/45 s; 60 °C/45 s; 74 °C/5 min) were performed in a thermal cycler (Cetus, Perkin-Elmer). The 5′-primer (GGGGATCCCATAAAGATGGAAAGACTCAGG) and the 3′-primer (GGTCTAGAAGAATCATGTCCAGCTGAAAGC) comprised *Bam*HI and *Xba*I restriction sites, respectively, to facilitate the cloning of the PCR product into the transfer vector pBacPAK8 (CLONTECH) downstream of the viral polyhedrin promoter to generate pBP-SBT1. Sequence analysis of pBP-SBT1 revealed the presence of two misincorporated nucleotides, both of which were found to be contained in a *Pml*I/*Xba*I fragment of pBP-SBT1. To eliminate the misincorporated nucleotides, this fragment was replaced with the corresponding fragment of the original *Le*SBT1 cDNA. Recombinant baculovirus (*Autographa californica* nuclear polyhedrosis virus) was generated by homologous recombinantion during co-transfection of *Sf*21 cells with pBP-SBT1 and linearized viral DNA (BacPAK6 baculovirus expression system, CLONTECH) according to the vendor's instructions. After 4 days at 28 °C, the culture supernatant was harvested and screened for viable virus by plaque assay (28). Individual viral plaques were isolated and used to infect insect cell cultures, which were subsequently tested for the expression of *Le*SBT1 by SDS-PAGE/Western blot analysis. Three monoclonal cultures out of five expressed recombinant *Le*SBT1. One of the recombinant viruses was chosen and amplified in subsequent cultures of 3, 15, and 30 ml (monolayers) and 50, 100, and 400 ml (cell suspensions) of serum-free medium according to standard protocols (28). The resulting 380 ml of high titer virus stock contained 8×10^7 plaque-forming units/ml.

*Overexpression and Purification of LeSBT1—Sf*21 cells (4×10^8) were collected by centrifugation ($200 \times g$), resuspended in 400 ml of fresh serum-free culture medium, and infected with 2×10^9 (multiplicity of infection of 5) recombinant virus particles from the high-titer virus stock (*cf.* previous paragraph). After 72 h of incubation, the culture was chilled to 4 °C, and the culture supernatant was separated from cells and membrane fragments by centrifugation. The $100{,}000 \times g$ supernatant was subjected to a fractionated ammonium sulfate precipitation from 50% to 90% saturation. The precipitate was desalted and transferred to buffer A (50 mM MES/sodium acetate, pH 5.8) using a HighTrap desalting column on the Äkta Explorer chromatography system (Amersham Pharmacia Biotech). All chromatographic steps were performed at room temperature. The protein sample was subjected to cation exchange chromatography on Resource S (1 ml column volume, Amersham Pharmacia Biotech) equilibrated in buffer A. Protein was eluted using a linear NaCl gradient (0–75 mM in 10 ml buffer A). Pooled fractions were subjected to final anion exchange chromatography on MonoQ (1-ml column volume, Amersham Pharmacia Biotech) equilibrated in 50 mM BisTris/Tris pH 7.0 (buffer B). A linear NaCl gradient (0–300 mM in 24 ml of buffer B) was used for elution of bound protein. Since no convenient assay for *Le*SBT1 activity was available, SDS-PAGE/Western blot analysis using the *Le*SBT1 antiserum (1:1000) and a goat anti-rabbit IgG alkaline phosphatase conjugate (1:3000; Bio-Rad) as the primary and secondary antibodies was used throughout the purification procedure to assay fractions for the presence of *Le*SBT1 protein.

MALDI-TOF Mass Spectometry Assay for LeSBT1 Activity—The activity of *Le*SBT1 was routinely assayed with glucagon as the substrate. Processing of glucagon was followed by MALDI-TOF mass spectrometric analysis of the peptide fragments generated. In standard assays, the reaction was performed in a total volume of 1 μl of reaction buffer (25

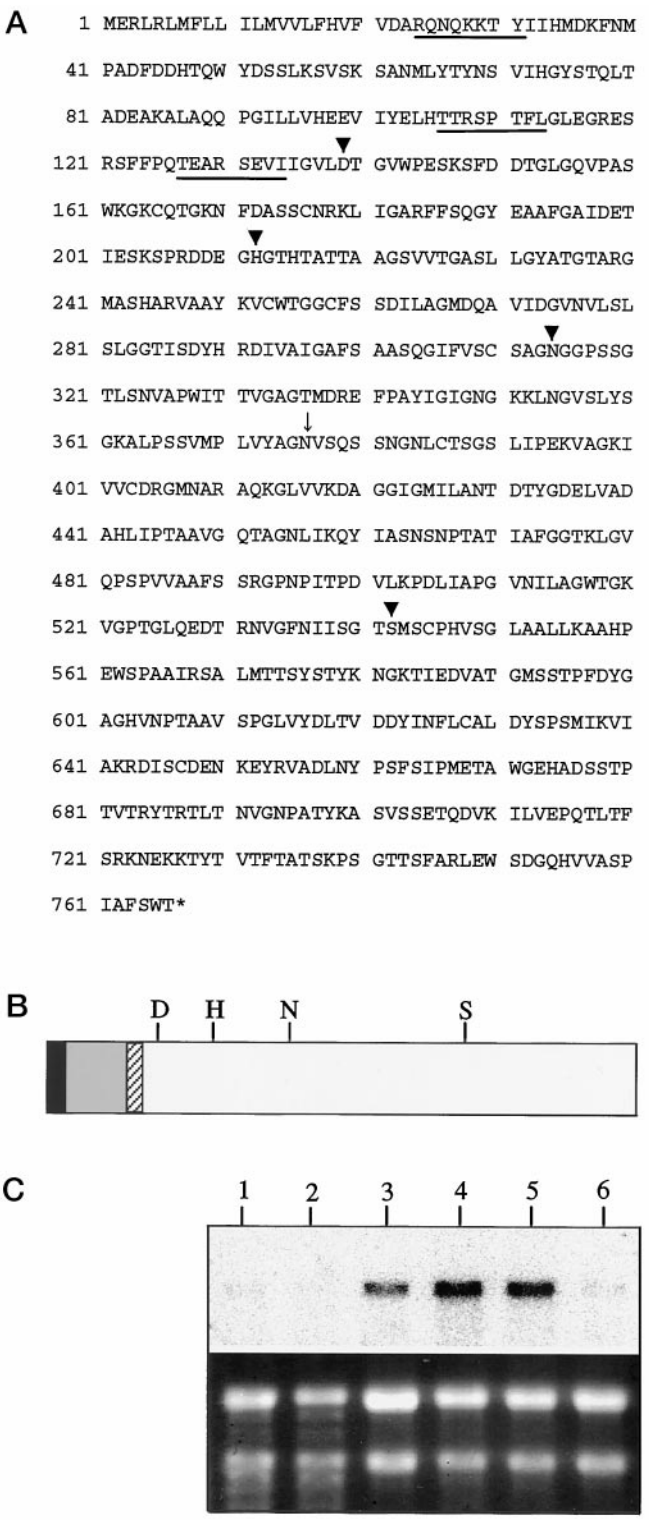

FIG. 1. **Primary structure and expression of *Le*SBT1.** *A*, the amino acid sequence of *Le*SBT1 deduced from its cDNA is shown. Stretches of amino acids that were identified by NH_2-terminal sequence analysis of various forms of *Le*SBT1 are *underlined*. The catalytically important residues conserved in subtilisin-like proteases are indicated (▼), as is a potential *N*-linked glycosylation site (↓). *B*, schematic representation of the *Le*SBT1 primary structure. The catalytic domain of mature *Le*SBT1 (*white box*) is preceded at the NH_2 terminus by a signal peptide, the prodomain, and an autoinhibitory peptide (*black*, *shaded*, and *hatched boxes*, respectively). The relative positions of the catalytically important Asp (D), His (H), Asn (N), and Ser (S) residues are indicated. *C*, Northern blot analysis of *Le*SBT1 expression. Steady state transcript levels of *Le*SBT1 were analyzed in cotyledons (*lane 1*), leaves (*lane 2*), roots (*lane 3*), stems (*lane 4*), flowers (*lane 5*), and cultured cells (*lane 6*) of tomato plants. Five μg of total RNA was hybridized on RNA gel blots to the radiolabeled *Le*SBT1 cDNA. A duplicate gel was stained with ethidium bromide as a control of RNA loading.

mM MES/acetate pH 6.0, 2 mM $CaCl_2$) on the MALDI sample plate. Unless otherwise indicated, the substrate (50 μM) was used in a 200-fold molar excess over the protease (250 nM) and the reaction was allowed to proceed for 5 or 15 min at room temperature. The reaction was quenched by addition of 0.5 μl 0.5% (w/v) trifluoroacetic acid, and the solution was mixed with 3 μl of the crystallization matrix (α-cyano-4-hydroxy-*trans*-cinnamic acid, prepared according to Beavis *et al*. (Ref. 29)), and mass spectra were recorded with a Voyager Elite mass spectrometer using the reflectron mode for increased mass accuracy. Peptide masses were further analyzed using the program PAWS version 8.1.1 (freeware edition for MacOs 7.5, copyright ProteoMetrics, 1997). For time course experiments, the reaction was performed in 1.5-ml microcentrifuge tubes, and aliquots were taken at the appropriate reaction time, quenched, and analyzed as before.

RESULTS

Molecular Cloning of LeSBT1—The screening of a tomato flower cDNA library (4×10^5 plaque-forming units) with a PCR-generated probe for tomato subtilases resulted in the isolation of 16 positive λ phage clones. Partial sequence analysis revealed that seven of the cDNA clones had sequences similar to subtilisin-related proteases. The longest cDNA was chosen for further analysis. Both strands of the 2689-base pair cDNA (accession no. X98929) were sequenced, and the cDNA was found to be derived from the *Le*SBT1 gene described previously (18). The *Le*SBT1 cDNA contained an open reading frame of 2298 base pairs (nucleotides 140–2437) with the capacity to code for the *Le*SBT1 protein of 766 amino acids. The deduced amino acid sequence (Fig. 1*A*) exhibits strong similarity to subtilases from tomato and other plant species (7, 12–18). Furthermore, the conceptual translation indicates that *Le*SBT1 is synthesized as a preproprotein. Computer analysis (SignalP version 1.1) predicts a 23-amino acid signal peptide for secretion at the amino terminus. Additional targeting signals for subcellular compartments were not detected, consistent with the possibility that *Le*SBT1 is an extracellular protease. Subtilases typically comprise a prodomain, which assists in the folding of the protease and serves as an intramolecular inhibitor of enzymatic activity (1, 30). For cucumisin (7), P69 (31), and the lily subtilase lim9 (12), the NH_2 termini of the mature enzymes have been determined and were found to be well conserved, beginning with an invariable pair of threonine residues. This sequence motif is also found in *Le*SBT1 (Fig. 1*A*), suggesting that *Le*SBT1 is a preproprotein comprising a 23-amino acid NH_2-terminal signal peptide, a prodomain of 82 amino acids, and a catalytic domain in which the amino acids of the catalytic triad (Asp^{139}, His^{212}, Ser^{542}), as well as the stabilizing Asn^{314}, are conserved (Fig. 1*B*). The *Le*SBT1 sequence contained a single consensus site (Asn-*X*-(Ser/Thr)) for potential *N*-linked glycosylation (Asn^{376}, *cf*. Fig. 1*A*). In insect cell-expressed *Le*SBT1, however, we failed to detect any protein bound sugar residues using the DIG glycan detection kit (Roche Diagnostics; data not shown). Northern blot analysis (Fig. 1*C*) revealed the presence of a single class of transcripts for *Le*SBT1 in roots, stems, and flowers of tomato plants. The transcript was hardly detectable in cotyledons, leaves, and suspension-cultured cells.

Overexpression and Purification of LeSBT1—A recombinant baculovirus (*A. californica* nuclear polyhedrosis virus) was generated carrying the open reading frame of the *Le*SBT1 cDNA under control of the polyhedrin promoter, which is active in the late phase of viral infection. The time dependence of *Le*SBT1 expression was analyzed in *Sf*21 cell monolayer cultures (Fig. 2). Two forms of the recombinant *Le*SBT1 protein, which differed in their apparent molecular masses, were found to accu-

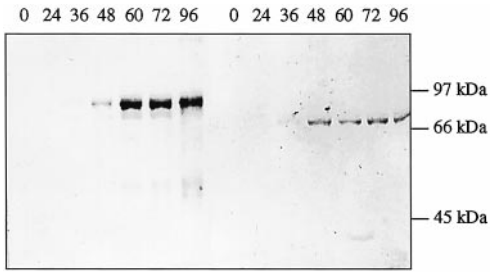

FIG. 2. **Expression of *Le*SBT1 in *SF*21 cell cultures.** *SF*21 cell cultures (9.6 cm^2, monolayers) were inoculated with recombinant baculovirus. At the time points (hours) indicated, the cells were resuspended, separated from the medium by centrifugation, and lysed by sonication. The insoluble protein fraction was collected by centrifugation (1000 × *g*), and 1/50 of the fraction was analyzed by SDS-PAGE/Western blot analysis (*left panel*). Likewise, the analysis of soluble proteins in the 100,000 × *g* supernatant of the culture medium (1/20 of total protein) is shown on the *right panel*. The protein gel blot was immunodecorated with an antiserum (1:1000) raised against a COOH-terminal fragment of *Le*SBT1.

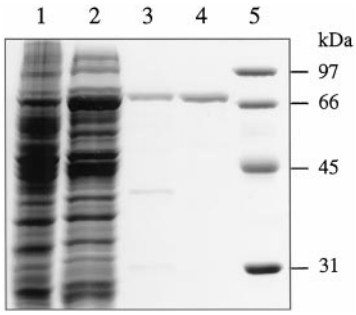

FIG. 3. **Purification of 73-kDa *Le*SBT1 from *Sf*21 cell suspension cultures.** A Coomassie Brilliant Blue-stained SDS-PAGE gel is shown. The medium of *Sf*21 cell suspension cultures (600 ml) was harvested 72 h after infection. Proteins in the 100,000 × *g* supernatant (*lane 1*, 20 μg) were subjected to fractionated ammonium sulfate precipitation (50–90% saturation; *lane 2*, 20 μg). After desalting, the proteins were further separated by cation and anion exchange chromatography. *Lanes 3* and *4* were loaded with 1 μg of the pooled protein fractions obtained after chromatography on ResourceS and MonoQ, respectively. The protein samples in *lanes 1–4* represent 1/3000, 1/1200, 1/1500, and 1/1000 of the total protein in the respective fraction. Protein molecular weight standards (Amersham Pharmacia Biotech) are shown in *lane 5*.

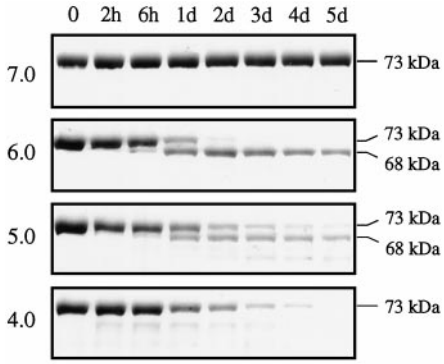

FIG. 4. **pH-dependent processing of 73-kDa *Le*SBT1.** 73-kDa *Le*SBT1 (0.2 mg/ml in 50 mM BisTris/Tris, pH 7.0) was dialyzed at 4 °C against 50 mM sodium acetate/acetic acid pH 6.0, 5.0, or 4.0 as indicated on the *left margin* of the figure, or else it was stored at 4 °C. Four-μl aliquots, corresponding to 800 ng of the initial protein concentration, were taken at the time points indicated in hours (*h*) and days (*d*), shock-frozen in liquid nitrogen, and stored at −80 °C. Samples were then analyzed on a Coomassie Brilliant Blue-stained SDS-PAGE gel. The positions of 73-kDa *Le*SBT1 and 68-kDa *Le*SBT1 are indicated.

mulate within the cells and in the culture supernatant, respectively. The intracellular form with an apparent molecular mass of 84 kDa first appeared 48 h after infection and was found to be insoluble. The NH_2-terminal amino acid sequence of the protein was determined (RQNQKKTY, indicated in Fig. 1*A*) and was found to correspond to the *Le*SBT1 proprotein, lacking the predicted 23-amino acid signal peptide. The less abundant secreted form of *Le*SBT1 with an apparent molecular mass of 73 kDa was first detectable in the culture medium at 36 h after viral infection, and highest levels were observed after 72 h. The NH_2-terminal amino acid sequence of this polypeptide (TTRSPTFL, indicated in Fig. 1*A*) corresponded to the amino terminus of the catalytic domain, which appears to be well conserved in plant subtilases (18). For purification of the extracellular form of *Le*SBT1, *Sf*21 suspension cell cultures in serum-free medium were infected with the recombinant baculovirus and the medium was collected 72 h after infection. The purification procedure involved fractionated ammonium sulfate precipitation, followed by cation and anion exchange chromatography. The progress of purification resulting in a homogeneous preparation of *Le*SBT1 is shown in Fig. 3. One liter of cell culture yielded 1.3 - 1.7 mg of the pure 73-kDa form of *Le*SBT1.

pH Dependence of LeSBT1 Zymogen Processing—The purified 73-kDa form of *Le*SBT1 did not exhibit proteolytic activity when assayed at pH 5.0–9.0 with various fluorogenic peptide conjugates, or glucagon as the substrates, and it was found to be stable at pH 7.0 and above. However, upon incubation at a pH between 5.0 and 6.0, a smaller protein was formed at the expense of the 73-kDa form of *Le*SBT1 (Fig. 4). The NH_2-terminal amino acid sequence of the newly formed 68-kDa protein (TEARSEVI, *cf.* Fig. 1*A*) indicates that this protein is a processed form of *Le*SBT1, lacking an additional 21 amino acids from the amino terminus. The lack of any detectable proteolytic activity in the initial protein preparation suggests that the formation of the 68-kDa *Le*SBT1 is the result of an autocatalytic processing event. At pH 6.0, 68-kDa *Le*SBT1 was formed most efficiently and was found to be rather stable. At pH 4.0 and 5.0, however, the 73-kDa as well as the 68-kDa forms of *Le*SBT1 were unstable, most likely due to autolytic degradation (Fig. 4). The formation of 68-kDa *Le*SBT1 could be correlated to the development of proteolytic activity using the 29-amino acid peptide glucagon as the substrate. Therefore, the 68-kDa form of *Le*SBT1 is considered to be the active protease, while the 73-kDa form is an inactive zymogen. Apparently, the maturation of *Le*SBT1 involves three consecutive processing steps, *i.e.* the removal of the signal peptide, of the prodomain, and of an amino-terminal inhibitory peptide (*cf.* Fig. 1*B*).

Substrate Specificity of LeSBT1—The 68-kDa *Le*SBT1 was generated from the purified 73-kDa zymogen by incubation at pH 6.0 (*cf.* Fig. 4), and its substrate specificity was analyzed using glucagon as the substrate. In the standard assay, the protease was incubated with a 200-fold molar excess of glucagon and the peptide fragments generated were analyzed by MALDI-TOF mass spectrometry. The 29-amino acid peptide glucagon was preferentially processed carboxyl-terminal of Gln^{24} and Gln^{20}. After extensive incubation, the resulting peptides (glucagon-(1–24) and glucagon-(1–20)) were further processed carboxyl-terminal of Asp^{15}, resulting in glucagon-(1–15) as the final product (Fig. 5). A comparison of the amino acid sequences at the three observed processing sites (Table I) reveals a preference of 68-kDa *Le*SBT1 for Gln in the P_1 position of the substrate (the amino acid NH_2-terminal of the hydrolyzed peptide bond; nomenclature of Schechter and Berger (Ref. 32)), amino acids with aliphatic side chains are found in the P_2 position, while Ser is frequent in P_5. The amino acid sequence of *Le*SBT1 at the internal processing site for activation meets these criteria (Table I) consistent with the observed autocatalytic generation of 68-kDa *Le*SBT1 from 73-kDa *Le*SBT1. A

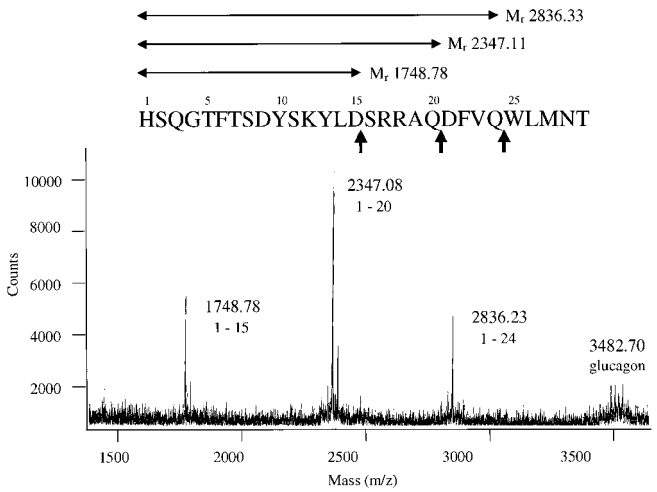

FIG. 5. **MALDI-TOF mass spectrometric assay of *Le*SBT1 activity.** A typical mass spectrum is shown obtained after incubation of glucagon (50 μM, M_r of 3482.79) with 68-kDa *Le*SBT1 (250 nM) as detailed under "Experimental Procedures." Three proteolytic fragments were observed with a M_r of 1748.78, 2347.08, and 2836.23, respectively. These masses correspond to those calculated for the fragments glucagon-(1–15), glucagon-(1–20), and glucagon-(1–24), as indicated at the top of the figure. For reference, the amino acid sequence of glucagon is shown and the observed sites of processing are indicated by the *arrows*.

fluorogenic tripeptide conjugate (Z-Gly-Ala-Gln-7-amido-4-methylcoumarin) was not cleaved by 68-kDa *Le*SBT1; neither were the peptide bonds carboxyl-terminal of Gln^3 in glucagon and Gln^4 in the oxidized insulin B chain, indicating that, for substrate recognition, more than four amino acids are required on the amino-terminal side of the hydrolyzed peptide bond. While *Le*SBT1 exhibited preference for Gln in the P_1 position of its substrate, this residue was not absolutely required. Cleavage of glucagon-(1–24) to yield glucagon-(1–15) indicates that Asp is tolerated in the P_1 position. However, cleavage carboxyl-terminal of Asp and accumulation of glucagon-(1–15) was slow except at pH 4.5 to 4.0 (Fig. 6), suggesting that the carboxyl of the Asp side chain has to be in its undissociated (protonated) form. In addition to Asp, Leu was tolerated in the P_1 position since processing of the oxidized insulin B chain was observed at a single site, carboxyl-terminal of Leu^{15} (Table I).

pH Dependence of LeSBT1 Activity—The mass spectrometric assay with glucagon as the substrate for 68-kDa *Le*SBT1 was employed to obtain a qualitatative estimation of reaction rates. In this assay, the relative abundance of glucagon and its cleavage products after a given reaction time was determined and used as a measure of *Le*SBT1 activity. The protease was found to be inactive at pH 7.0 and above. Cleavage of glucagon was first detected at pH 6.0. As the pH was lowered further, there was increasing degradation of glucagon, indicating an increase in proteolytic activity being highest between pH 5.0 and 4.0 (Fig. 6). Concomitant with the degradation of glucagon, its 24- and 20-amino acid degradation products (glucagon-(1–24) and glucagon-(1–20)) were formed. When 68-kDa *Le*SBT1 activity was highest and all the glucagon had been consumed, these products were further processed to yield glucagon-(1–15) (Figs. 5 and 6).

When 73-kDa *Le*SBT1 was analyzed under the same conditions, it was found to be devoid of enzymatic activity between pH 9.0 and 5.0. At pH 4.0, however, glucagon was efficiently degraded (data not shown). Apparently, processing of the NH_2-terminal 21 amino acids is not required for catalytic activity at pH 4.0. This finding is consistent with the observation of autolytic degradation of 73-kDa *Le*SBT1 at pH 4.0. At this pH, processing of the 73-kDa form to yield 68-kDa *Le*SBT1 was not detectable; nonetheless, the 73-kDa form was autolytically degraded and lower molecular weight protein bands were observed during SDS-PAGE (Fig. 4).

*Le*SBT1 tolerated a 20-min incubation at 50 °C and was partially inactivated at 60 °C, while complete loss of activity during this period was observed at higher temperatures. None of the tested inhibitors (1 mM phenylmethylsulfonyl fluoride, 0.15 mM N^{α}-*p*-tosyl-L-lysine chloromethyl ketone, 0.3 mM L-1-tosylamido-2-phenylethyl chloromethyl ketone, 0.3 μM aprotinin, 5 mM dithiothreitol, 2 mM $ZnCl_2$) caused a significant reduction in proteolytic activity.

DISCUSSION

Processing of PreproLeSBT1—The conceptual translation of the *Le*SBT1 cDNA derived from tomato flowers revealed typical features of subtilisin-like proteases, including the arrangement of the catalytically important amino acid residues and the three functional domains of a preproprotein (*cf.* Fig. 1; Ref. 1). The existence of both the pre- and prodomains was confirmed experimentally. Upon overexpression in insect cells, an insoluble form of *Le*SBT1 with an apparent molecular mass of 84 kDa accumulated intracellularly. NH_2-terminal sequence analysis revealed that the predicted 23-amino acid signal peptide of *Le*SBT1 for co-translational targeting to the secretory pathway was missing, indicating that the insoluble protein accumulated within the endoplasmic reticulum. Similarly, intracellular accumulation of insoluble murine PC1/PC3 was observed after overexpression in baculovirus-infected insect cells (33). The insoluble form of the two proteins is likely to be an artifact of the overexpression system. The strong polyhedrin promoter, used to control the expression of both *Le*SBT1 and PC1/PC3, is active during the late phase of viral infection, when the secretory machinery of the host cell may already be impaired. Thus, the synthesis of *Le*SBT1 may well exceed the capacity of the secretory pathway, resulting in the accumulation of pro*Le*SBT1 and the formation of inclusion bodies. This view is supported by the fact that accumulation of intracellular 84-kDa *Le*SBT1 was observed with a time lag of 1 day, as compared with the secreted 73-kDa form.

The secreted form of *Le*SBT1 with an apparent molecular mass of 73 kDa during SDS-PAGE (calculated M_r of 69066) was purified to homogeneity. Its NH_2-terminal amino acid sequence beginning with a pair of threonine residues is highly similar to the amino termini that were determined for the mature, active cucumisin (7), P69 (15, 31), lim9 (12), and macluralisin (11). Apparently, the processing site at the junction between the prodomain and the catalytic domain is well conserved in plant subtilases (18). This is also true for mammalian PCs in which a cluster of basic amino acids is found at the COOH terminus of the prodomain. The amino acid sequence at the domain junction reflects the substrate specificity of the respective PC allowing processing of the prodomain by an autocatalytic intramolecular reaction in many PCs (34–38). The observed processing of the prodomain of insect cell-expressed *Le*SBT1 suggests that this may also be an autocatalytic process, or else, a processing endoprotease with a substrate specificity similar to that of the respective plant enzyme must be postulated within the secretory pathway of insect cells.

In contrast to other plant subtilases (7, 11, 12, 15), processing of pro*Le*SBT1 did not result in zymogen activation. Maturation of *Le*SBT1 required the additional processing of 21 amino acids from its amino terminus. Zymogen activation was observed in a homogeneous preparation of 73-kDa *Le*SBT1 and, thus, is likely to be the result of an autocatalytic reaction. This process was found to be strictly pH-dependent. While 73-kDa *Le*SBT1 was stable at pH 7.0, 68-kDa *Le*SBT1 (calculated M_r of 66670) was formed at pH 6.0 concomitant with the generation

TABLE I
Substrate specificity of LeSBT1

For glucagon and the oxidized insulin B chain, processing sites were determined by MALDI-TOF mass spectrometric analysis of cleavage products. The internal processing site for activation in *Le*SBT1 was identified by N-terminal sequence analysis of 68-kDa *Le*SBT1. The amino acid sequences are aligned with respect to their sites of processing between the P_1 and the P'_1 residues (nomenclature of Schechter and Berger (Ref. 32)).

Substrate	P_5	P_4	P_3	P_2	P_1	P'_1	P'_2
Glucagon	Ser	Arg	Arg	Ala	Gln	Asp	Phe
Glucagon	Gln	Asp	Phe	Val	Gln	Trp	Leu
Glucagon	Ser	Lys	Tyr	Leu	Asp	Ser	Arg
73-kDa *Le*SBT1	Ser	Phe	Phe	Pro	Gln	Thr	Glu
Insulin B chain	Leu	Val	Glu	Ala	Leu	Tyr	Leu

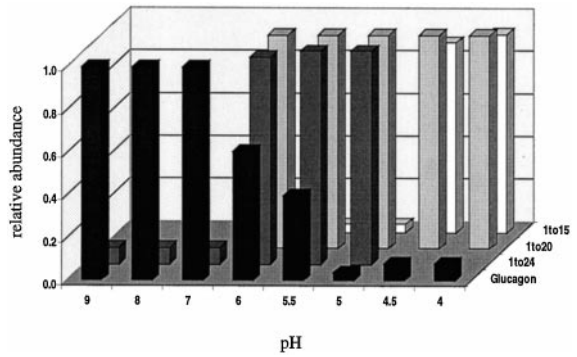

FIG. 6. **pH dependence of *Le*SBT1 activity.** 68-kDa *Le*SBT1 was incubated with glucagon for 5 min at room temperature at pH values ranging from 4.0 to 9.0. The buffers used were sodium acetate/acetic acid for pH 4.0–6.0, BisTris/Tris for pH 7.0, and Tris/HCl for pH 8.0–9.0, each at 50 mM. Proteolytic degradation products were analyzed by MALDI-TOF mass spectrometry. In each experiment, the most abundant peptide was normalized to 1 (relative units) and the relative abundance of other fragments is shown in fractions thereof.

of proteolytic activity. Apparently, *Le*SBT1 is activated upon targeting to an acidified compartment, which likely is the apoplast, *i.e.* the continuous extracellular space consisting mainly of the plant cell wall. The pH dependence of *Le*SBT1 maturation may thus provide a mechanism to protect proteins in early compartments of the secretory pathway from its proteolytic activity. Most of the mammalian PCs mature at neutral pH in the endoplasmic reticulum (36, 39–42). However, as observed for *Le*SBT1, cleavage of the prodomain in PCs does not necessarily result in the activation of the enzyme. The activation of furin, for example, requires its exposure to the mildly acidic environment of the trans-Golgi network. The initial cleavage of the furin prodomain occurs at neutral pH in the early secretory pathway. The prodomain is not released but rather remains bound to the enzyme as an inhibitor of its activity. In the trans-Golgi network, at pH 6.0, the furin propeptide is cleaved internally a second time and the dissociation of the resulting fragments is facilitated (41, 43, 44). A pH-dependent zymogen processing has also been described for PC2 (36). ProPC2 is stable at neutral pH, but rapid autocatalytic processing was observed at pH 5.0, indicating that proPC2 maturation is controlled by the decreasing pH gradient along the secretory pathway (38, 45). Murine PC1/PC3 provides another example of pH-dependent activation of a proprotein convertase. For PC1/PC3, carboxyl-terminal processing concomitant with an increase in proteolytic activity has been observed at acidic pH levels (33). The complex maturation pathway of *Le*SBT1 appears to represent a novel variation on the common theme of pH-controlled zymogen activation in subtilisin-like proteases.

pH Dependence of LeSBT1 Activity—The 68-kDa *Le*SBT1 was found to be inactive at pH 7.0 and above, and its activity increased with decreasing pH between pH 6.0 and 4.0. Its acidic pH optimum is consistent with its presumed localization in the apoplast of the plant cell and opposed to that of other plant subtilases (8, 10–12, 46). In contrast to 68-kDa *Le*SBT1, the 73-kDa form of *Le*SBT1 was inactive between pH 9.0 and 5.0 but showed proteolytic activity at pH 4.0. Apparently, processing of the NH_2-terminal 21 amino acids is not required for zymogen activation at this acidic pH. In an attempt to rationalize these results, we propose a role for the NH_2 terminus of *Le*SBT1 as an intramolecular inhibitor of protease activity. Possibly, the NH_2 terminus of *Le*SBT1 binds to the active site of the enzyme and thus prevents access of potential substrates to the substrate binding pocket. The interaction with the substrate binding site may well be pH-dependent, resulting in the release of the NH_2 terminus and in free accessibility of the active site at pH 4.0. A similar mechanism for pH-dependent regulation of catalytic activity has been described for cathepsin D (47). Cathepsin D is a lysosomal aspartic proteinase involved in animals in protein turnover as well as in selective protein processing. Mature cathepsin D is inactive at neutral pH but, upon acidification, fully reversible activation is observed. Recently, the crystal structure of the inactive, mature enzyme has been elucidated at pH 7.5. The structure revealed that the NH_2-terminal 16 amino acids insert into the active site cleft of cathepsin D thus preventing access of substrates and inhibitors (47). Upon acidification, a conformational change occurs releasing the NH_2-terminal autoinhibitory peptide and restoring accessibility of the active site (47). A similar mechanism of reversible non-proteolytic activation at acidic pH has also been described for prorenin and pepsinogen (see Ref. 48, and references therein). Likewise, for prophytepsin, the zymogen of a barley aspartic proteinase, the NH_2-terminal 13 amino acids were shown to contribute to the flap which blocks the active site of the enzyme (49).

Substrate Specificity of LeSBT1—Using glucagon as the substrate, 68-kDa *Le*SBT1 was shown to preferentially hydrolyze the peptide bond on the carboxyl side of Gln residues. Gln in the P_1 position, however, is not the only parameter required to describe the substrate specificity of *Le*SBT1. Gln is present in the 3rd and 4th positions of glucagon and the oxidized insulin B chain, respectively. However, these bonds were not hydrolyzed by *Le*SBT1 and neither was a fluorogenic tripeptidyl amino-4-methylcoumarin conjugate with Gln in the ultimate position. The data indicate that *Le*SBT1 has an extended substrate specificity pocket. A similar observation was made for mammalian SKI-1, which, like *Le*SBT1, belongs to the pyrolysin family of subtilases (1, 20). SKI-1 does not cleave small fluorogenic substrates of sequences resembling the physiological substrate pro-brain-derived neurotrophic factor (20).

The substrate specificity exhibited by *Le*SBT1 is clearly different from that of mammalian PCs with the general recognition motif (Arg/Lys)-X_n-(Arg/Lys) ↓ , where n is 0, 2, 4, or 6 (2). It is also differs from the substrate specificity of mammalian pyrolysins (Arg-*X*-*X*-(Leu/Thr) ↓ , (19, 20) and it is clearly more refined than the specificity of other subtilisin-like proteases from plants. The plant subtilases cucumisin and macluralisin

were found to accept a broad range of substrates, and each cleaved the insulin B chain at eight different positions (8, 9, 11). Taraxalisin, a serine proteinase from dandelion that likely belongs to the subtilase family, hydrolyzed nine peptide bonds within the oxidized insulin B chain (10). Therefore, in contrast to hitherto described plant subtilases, *Le*SBT1 is not likely to serve a degradative function but may be involved in selective protein processing, as are the PCs and the pyrolysins SKI-1 and S1P in animals (2, 3, 19, 20).

It has been shown *in vitro* and in co-expression studies that many PCs are able to cleave common substrate proteins at oligobasic sequence motifs. *In vivo*, however, the range of substrates is much more restricted to those protein precursors that are co-localized with the respective protease in the same cell type and at the same time in development (2, 50, 51). Therefore, the highly regulated expression of PCs contributes to the exquisite *in vivo* substrate specificity of these proteinases. A similar situation may exist in tomato plants, where *Le*SBT1 expression was found to be restricted to roots, stems, and flowers. This pattern of expression is unique among all tomato subtilases (18). Clearly, the search for the physiological substrates of *Le*SBT1 will have to begin in the apoplast of the organs where its expression has been observed. The identification of these substrates will be indispensable for the future elucidation of *Le*SBT1 function *in vivo*.

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