Discovery of Substrate for Type I Signal Peptidase SpsB from Staphylococcus aureus*

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Based on the kinetic model of substrate phage proteolysis, we have formulated a strategy for best manipulating the conditions in screening phage display libraries for protease substrates (Sharkov, N. A., Davis, R. M., Reidhaar-Olson, J. F., Navre, M., and Cai, D. (2001) J. Biol. Chem. 276, 10788–10793). This strategy is exploited in the present study with signal peptidase SpsB from Staphylococcus aureus. We demonstrate that highly active substrate phage clones can be isolated from a phage display library by systematically tuning the selection stringency in screening. Several of the selected clones exhibit superior activity over a control, the best clone, IIIIRIII-8, showing >100-fold improvement. Because no conserved sequence features were readily revealed that could allow delineation of the active and unreactive clones, the sequences identified in five of the active clones were tested as synthetic decaamers, Ac-AGX₃GA-NH₂. Using electrospray ionization mass spectrometry, we show that four of these peptides can be cleaved by SpsB and that Ala is the P1 residue exclusively and Ala or Leu the P3 residue, in keeping with the (−3, −1) rule for substrate recognition by signal peptidase. Our successful screening with SpsB demonstrated the general applicability of the screening strategy and allowed us to isolate the first peptide substrates for the enzyme.

Bacterial type I signal peptidase is responsible for cleaving the signal peptide from precursor proteins, and its activity is an integral part of the export and maturation of secreted proteins in vivo. The essential function of the enzyme to bacterial cell viability has been demonstrated using genetic approaches with both Gram-positive and Gram-negative organisms (1–3), supporting the notion that the signal peptidase is potentially an antibacterial target (4). Drug discovery efforts with the enzyme, however, may be hampered by the lack of an effective in vitro assay employing a nonprotein substrate such as a peptide (4).

Our current understanding is that signal peptides are highly variable in sequence (5). Based on the studies carried out over the past 2 decades, it has been established that the recognition sites for signal peptidases lie between −6 and +1 in sequences encompassing the site of cleavage (6–12). Sequence conservation analyses of a large panel of naturally occurring signal peptides in bacteria and eukaryotes reveal that the predominant residue at the P1 site is Ala and that the predominant residues at the P3 site are large aliphatic residues (Leu, Ile, Val) as well as Ala and Ser, a consensus dubbed the (−3, −1) rule (9–11). The (−3, −1) rule also holds for the cleavage of engineered preproteins in vivo as well as in vitro (6–8, 12). The reaction of signal peptidases with synthetic peptides, on the other hand, is not as well explored as with protein substrates. For the signal peptidase LepB from Escherichia coli, the best characterized signal peptidase, Ala was found as the only residue permitted at the P1 site through single amino acid replacements of a peptide bearing the signal peptide sequence of the E. coli maltose-binding protein (13).

As demonstrated with the E. coli LepB enzyme, the catalytic efficiency of signal peptidase toward short peptide substrates is generally several orders of magnitude lower than toward polypeptides bearing the same protease recognition sequence (14–16). Various approaches including computational designs have been attempted with limited success in search of more highly functional peptides to serve as substrates for the E. coli enzyme (17–20). For instance, peptide libraries were created by incorporating randomized sequences into the signal peptide of TEM-1 β-lactamase, varying six amino acid residues between −4 and +2 positions around the signal peptidase cleavage site (19). Functional sequences were found to support the production of active TEM-1 but none better than the wild type. Reported more recently were combinatorial synthetic peptide libraries in which four positions, −4, −3, −2, and +2, were varied in the signal peptidase recognition sequence, and better than 10-fold improvements over the control were observed among the selected peptides (20).

One unsurpassed advantage of phage display over other combinatorial approaches is its capacity to generate a vast number of possible combinations. It is experimentally feasible to randomize up to eight amino acid residues in one library. Phage display has been successfully applied to proteases for selection and optimization of peptide substrates by way of optimizing the substrate phage (21–24). A good peptide substrate in turn would aid the development of protease assays in vitro. Recently, we reported that the proteolysis of substrate phage is a single exponential process and provided the kinetic basis for how to control the rate of proteolysis to ensure the success of substrate phage selection (25). The experimental design strategy we put forward is now exploited in the present study, where we applied it to the screening of an 8-mer phage display library with the type I signal peptidase SpsB from Staphylococcus aureus. By systematically tuning the screening stringency in the selection process, we discovered several active substrate phage clones. The sequences found in the most reactive clones were subsequently evaluated as synthetic peptides and characterized for their competency to serve as substrates...
for SpsB with respect to the substrate recognition and digestion kinetics.

**EXPERIMENTAL PROCEDURES**

**Materials**—The phage display library fTC-LIB-N8 used in this study was reported earlier (24). The recombinant signal peptidease SpsB, expressed in *E. coli*,1 was kindly provided by Monica Gevi and Birger Jansson of GlaxoWellcome Spa, Italy. The purified protein was stored in enzyme storage buffer (0.5% Triton X-100, 10% glycerol in phosphate-buffered saline, pH 7.4), and the enzyme concentration was calculated from the protein concentration.2 The biotinylated mouse monoclonal antibody mAB179 was prepared in house and kindly provided by Bruce Mortenson, and antibody mAB3-E7 was from a previous study (23). Pansorbin cells were purchased from Calbiochem. Phage clones were maintained and amplified using *E. coli* K01 cells (25). Synthetic peptides of >95% purity were purchased from SynPep Corp. (Dublin, CA) and were acetylated and amydinated at the N and C termini, respectively.

**Phage Library Screening, Phage Preparation, and Phage ELISA**—In round one of slicing I (see Table I for designation), 30 µl of the phage library fTC-LIB-N8 at 6.7 × 10^11 colony-forming units/ml were mixed with 25 µl of 175 µM signal peptidease SpsB and 195 µl of assay buffer (50 mM Tris-HCl, pH 9.0, 200 mM NaCl, 5 mM MgCl₂ and 0.375% Triton X-100). The negative control was the same except for the replacement of SpsB with 25 µl of enzyme storage buffer. After 24 h of incubation at 25 °C, 30 µl of 1% bovine serum albumin, 33 µl of 0.3% mg/ml mAb 179, and 10 µl of 1 mg/ml mAb3-E7 were added. After 30 min on ice, 100 µl of Pansorbin cells were added, and the reaction mixture was placed on a rotating mixer at 4 °C for 1 h. The mixture was centrifuged at 4 °C for 2 min, and the supernatant was recovered. A second addition of 100 µl of Pansorbin cells was made, and the Pansorbin cell adsorption step was repeated. The final supernatant was amplified overnight in *E. coli* K91 cells, and the phage particles were purified (see below). A small aliquot of the final supernatant solution was also used for titering on *E. coli* K91 cells. Round two of slicing I was then continued for 24 h with 30 µl of the amplified phage from round one, 25 µl of 175 µM SpsB, and 195 µl of assay buffer. The selection round three was continued similarly following the completion of round two. The incubation time of other slicing experiments is indicated in Table I.

Starting at selection round two, individual colonies were picked at random from the Petri dishes from the titering step and cultured in 12-ml LB medium containing 15 µg/ml tetracycline overnight at 37 °C to produce individual phage clones. The phage particles of each clone were precipitated from the supernatant following a general procedure described previously (23). Phage preparations were typically resuspended in ≤50 µl of TBS (150 mM NaCl and 50 mM Tris, pH 7.5). The phage titer of these preparations was determined by phage ELISA performed following a general procedure described previously (25). These individual clones were then screened for SpsB substrate activity.

**Activity Screening and Sequencing of Individual Phage Clones**—For the activity screening of individual clones, each clone was diluted in 40 µl of assay buffer containing 17.5 µM SpsB alongside 40 µl of diluted phage with no enzyme as a control. The dilution was determined according to the titer such that following a 50x dilution, the starting phage titer would produce an ELISA signal corresponding to the uppermost part of the “useful range” (25). The reaction was incubated at 25 °C, and 5-µl aliquots were typically removed at 10 min, 30 min, 1 h, and 2 h and diluted in 250 µl of 0.1% bovine serum albumin in phosphate-buffered saline to stop the reaction. 200 µl of the stopped reaction were transferred into ELISA plates and developed to check for the extent of cleavage. Clones showing ≥40% digestion in this time period were considered active, and the most active clones showed significant if not complete digestion within 10 min. Active clones were cultured overnight in 1 liter of LB medium containing 15 µg/ml tetracycline at 37 °C, and the purified phage particles were typically resuspended in 1 ml of phage storage buffer (25 µl Tris, pH 7.4, 20% glycerol, 1% sucrose) and then titered.

The top half of the clones resulting from all slicing experiments were further screened and ranked according to the digestion rate constant *k*<sub>[obs]</sub> obtained by following the complete digestion time course in the presence of 15 or 17.5 µM SpsB, because the single-exponential rate constant is a more precise measure of the substrate reactivity. The amount of substrate remaining at various time points was determined using the ELISA assay, and the data were fitted to Equation 1 as described previously using nonlinear regression (25) to determine *k*<sub>[obs]</sub>:

\[
\frac{[S]}{[S]_0} = Ae^{-k_{[obs]}t} + C \quad \text{(Eq. 1)}
\]

where [S<sub>0</sub>] represents the starting substrate concentration.

Phage DNA for sequencing was isolated from a 12-ml overnight culture using a commercially available M13 single-stranded DNA isolation kit, the Wizard M13 DNA Purification System, from Promega (Madison, WI). The sequencing primer was 5'-CAGTCTAGAAGTTTGCAGCTGG-3' which generated the sequence of the complementary antisense strand. The automated sequencing service was provided by Biotech Core (Palo Alto, CA).

**Kinetic Characterization of Single Substrate Phage Clones**—For the careful determination of proteolytic activity of the active phage clones, the digestion and ELISA were performed by automated means with a robotic system from Scitec Inc. (Ashland, MA) as previously described (25). The optimal dilution of phage in the digestion reaction was determined individually for each clone. The starting phage concentration was chosen such that following a 33.3-fold dilution in stop solution, it would produce an ELISA signal corresponding to the uppermost part of the “useful range” of the phage standard curve (25). The *k*<sub>[obs]</sub> was determined for five of the active clones at various SpsB concentrations, and the data were fitted to Equation 2, derived for a pseudo-first-order reaction model (25), to calculate the substrate and enzyme binding constant *k*<sub>i</sub>.

\[
k_{[obs]} = k_i[E]_o + k_{-1} \quad \text{(Eq. 2)}
\]

All enzymatic reactions were carried out at 25 °C.

**Mass Spectrometry Analysis of the Proteolysis of Synthetic Peptide**—The synthetic peptides were typically dissolved in Me<sub>2</sub>SO: peptides 1 and 6 to 10 mM, peptide 2 to 50 mM, peptide 3 to 25 mM, and peptides 4 and 5 to 100 mM (all based on solubility in Me<sub>2</sub>SO). To determine the cleavage site, 250 µg peptide was incubated with 17.5 µM SpsB in 200 µl of assay buffer at 25 °C for 16.5 h or otherwise as indicated, all containing 1% Me<sub>2</sub>SO in the final digestion reaction except for peptides 1 and 6, which had 2.5% Me<sub>2</sub>SO. The digestion reaction (digest) was stopped by adding an equal volume of stop solution (20% acetic acid plus 80% ACN), and the mixture was spun in a microcentrifuge at maximum speed for 10 min. In parallel, two control reactions were prepared similarly, one containing the enzyme only (SpsB only; see Fig. 3) and the other only peptide (peptide only). 5 µl of the postdigest were removed and analyzed using an LC/MS instrument from Agilent (Palo Alto, CA) consisting of a quadrupole ion analyzer (Agilent 1100 MSD), a binary pump, an autosampler, a temperature-controlled column compartment, and a diode array detector. The sample was injected onto a YMC Jsphere column and eluted with an ACN gradient from 10% ACN, 0.2% formic acid to 95% ACN, 0.2% formic acid in 5 min at a flow rate of 1 ml/min. The LC/MS instrument was operated in electrospray positive ionization mode, and the spectra of 200–2000 mass range were recorded. The acquired mass spectra were analyzed using the Agilent ChemStation software to search for the appearance of cleaved products. For each peptide, the expected molecular weight of products from all possible cleavage sites was calculated first, and the ions of the exact molecular weight were extracted from the mass spectrum. The products resulting from the actual digestion were then present in the digest spectrum and absent in the SpsB-only and peptide-only control spectra.

**Activity Assay of Peptide Substrates**—To quantitate the extent of digestion, 50 µl of the same supernatant used above for the LC/MS experiment were also analyzed using an HPLC instrument by Rainin (Emeryville, CA) equipped with a Dynamax UV detector. The separation was achieved on a Merckney-Nagel ET 250/3 Nucleosil 100-5C18 column using an ACN gradient from 10% ACN, 0.1% trifluoroacetic acid to 100% ACN, 0.1% trifluoroacetic acid in 30 min at a flow rate of 0.5 ml/min. The percentage of digestion was calculated by taking the ratio of the area of the eluted substrate peak from the digest sample and that from the peptide-only control.

The activity of peptides 2 and 4 was further assayed under the steady-state conditions using an end point method. The substrate was diluted in Me<sub>2</sub>SO, and the final substrate concentration in the assay was 5–100 and 2–500 µM for peptides 2 and 4, respectively. The Me<sub>2</sub>SO concentration was kept constant at 1%. The diluted substrate was incubated with 17.5 µM SpsB at 25 °C for 16 h for peptide 2 and with 1 µM SpsB for 90 or 290 min for peptide 4. Following the addition of an equal volume of stop solution and centrifugation, 50 µl of the superna-

1 Monica Gevi and Birger Jansson, unpublished results.
2 The abbreviations used are: ELISA, enzyme-linked immunosorbent assay; ACN, acetonitrile; HPLC, high-pressure liquid chromatography; LC/MS, liquid chromatography/mass spectrometry. 

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The amount of substrate remaining was determined at different time points in the presence (●) and absence (○) of 17.5 μM SpsB. The data collected in the presence of SpsB were fitted to Equation 1, yielding $k_{\text{obs}} = (1.17 \pm 0.85) \times 10^{-3}$ min$^{-1}$, and the line represents the nonlinear curve fit to Equation 1.

**RESULTS**

**Positive Control Substrate**—Signal peptidase SpsB from *S. aureus* is known to complement LepB-deficient *E. coli* mutants (1), indicating that it may recognize and correctly process substrates bearing a cleavage sequence of the *E. coli* enzyme. A control clone SigPep1 was thus created, containing the cleavage sequence of the *E. coli* maltose-binding protein (see Table II). Using the phage ELISA that we developed to determine the concentration of intact substrate phage (25), we found that 17.5 μM SpsB was able to cleave SigPep1 at 25 °C with a $t_{1/2}$ of about 10 h (Fig. 1). The course of digestion was single-exponential, consistent with our previous observation with other proteases (25). The experiment demonstrated that the activity of SpsB could be detected and assayed properly even for a substrate with a poor reactivity.

**Phage Library Screening**—The phage display library fTC-LIB-N8 was screened according to the procedure described under “Experimental Procedures.” Five independent screening experiments, slicings I–V, were performed, and the results are summarized in Table I. The screening experiments are arranged in Table I in the order of increased screening stringency, which was controlled by varying the incubation time in each round of the selection. The incubation was kept constant in all selection rounds in every experiment except for slicing III, where it was shortened from 24 to 1 h after the first two rounds. As expected (25), the number of active clones declined as the selection became more stringent. The activity of each clone picked was scored according to the extent of substrate cleavage upon incubation with 17.5 μM SpsB at 25 °C for up to 2 h, and the clones showing $>40\%$ cleavage were considered active. According to this criterion, only a small fraction of the total colonies screened was deemed active, and the ratio became even smaller without a 24-h incubation in all rounds of selection, implying that the library contained only a small population of potential substrates for SpsB.

The reactivity of 11 active clones resulting from the screening was analyzed further. We followed their complete digestion time course in the presence of 15 or 17.5 μM SpsB using the phage ELISA and found that the digestion process was single-exponential for all of the clones (data not shown). The first-order rate constants $k_{\text{obs}}$ are reported in Table II.

**Pseudo-first-order Kinetics of Substrate Phage Digestion**—The four most active clones SIIIRIII-8, SIRIII-5, SIIIBRIII-11, and SIRIII-30 were subjected to full kinetic characterization along with clone SIRIII-35, which was selected because the sequence in its variable region was closely related to that in clone SIIIIIIBRIII-11. The proteolysis kinetics were investigated as a function of the SpsB enzyme concentration. Shown in Fig. 2A is an example with SIRIII-5 to illustrate the single-exponential disappearance of the substrate and the dependence of the first-order rate constant on enzyme concentration. The data shown in Fig. 2B indicate a linear function of the enzyme concentration dependence for all five clones, which conforms with our previous conclusion that the reaction between substrate phage and enzyme obeys pseudo-first-order kinetics (25). Only one data point was obtained for the control SigPep1 with 15 μM SpsB, and, for the purpose of comparison, it was included in Fig. 2B. It is clear that the five clones we characterized demonstrate a superior reactivity over the control. Based on the values of $k_{\text{obs}}$ with 15 μM SpsB as reported in Fig. 2B, we found a 54-, 23-, 16-, and 9-fold improvement over SigPep1 for clones SIIIRIII-5, SIIIBRIII-11, SIRIII-30, and SIRIII-35, respectively. SIIIRIII-8 shows the most improvement, estimated at 112-fold over SigPep1 (for SIIIRIII-8 and SIIIBRIII-11, $k_{\text{obs}}$ values with 15 μM SpsB were extrapolated from the linear fit in Fig. 2B). The relative reactivity of these clones was also manifested in the slope of the linear curve fit (Table III), *i.e.*, the binding rate constant $k_1$ between the substrate and enzyme (25).

**Sequence Analysis**—The phage DNA was isolated from the control SigPep1 and the 11 active phage clones shown in Table II. The portion of DNA encompassing the variable region was sequenced, and the amino acid sequence was translated from the nucleotide sequence. 10 additional clones were also picked at random, and their phage DNA was purified and sequenced after it was shown that they did not exhibit any appreciable digestion by 17.5 μM SpsB following a 2-h incubation. The sequencing results for both active and unreactive clones are shown in Table II. We found, examining the sequences, that most clones contained at least one Ala residue in the variable region regardless of their reactivity. The occurrence of charged residues and the distribution of other residues seemed indistinguishable between the two groups of clones and might appear rather to reflect the abundance of available codons encoding the residues. For instance, the frequent occurrence of Arg in almost every sequence might result from the fact that it can be encoded by six codons, as can Leu and Ser, and might have no real significance in rendering certain reactivity to the sequence. Therefore, no sequence conservation was evident that would allow for deduction of a consensus sequence or for the sequence of active clones to be aligned in a manner that would be reflective of a plausible cleavage pattern.

**Site-specific Cleavage of Synthetic Peptides**—Because the amino acid sequence analysis offered no plausible clues to deciphering conserved features among the active phage clones, we decided to test the sequences found in the five fully characterized clones in synthetic peptides. In designing the peptides, we included the eight residues present in the variable region and two additional residues on each side so that the...
### Table I

Results of five independent phage display library screening experiments

| Experiment | Selection | Colonies picked | Active clones |
|------------|-----------|-----------------|---------------|
| Slicing I  | 3 rounds of 24-h incubations | 12 from round II | 3             |
|            |           | 36 from round III | 13            |
| Slicing V  | 3 rounds of 24-h incubations | 24 from round III | 2             |
| Slicing III| Rounds I and II of 24-h incubations, rounds III & IV of 1-h incubations | 24 from rounds III and IV | 1             |
| Slicing IV | 3 rounds of 5-h incubations | 72 from round III | 2             |
| Slicing IIB| 4 rounds of 0.5-h incubations | 24 from round II | None          |
|            |           | 48 from round IV | None          |

*The selection was performed as described under “Experimental Procedures.”
*The clones were determined to be active if ≥40% of substrate was cleaved by 17.5 µM SpsB within 2 h of the incubation at 25 °C.

### Table II

Amino acid sequence and reactivity of selective phage clones

The amino acid sequence, represented using a three-letter code, was translated from the nucleic acid sequence of purified phage DNA. Italicized are residues found in the randomized region, and shaded are those in the flanking sequences that do not match the sequence in the library fTC-LIB-N8 construct. Underlined are sequences later incorporated into synthetic peptides (see Table III).

#### Clones

| Clones    | Translated amino acid sequence | Activity |
|-----------|--------------------------------|----------|
| fTC-LIB-N8| ser gly gly ala gly N1 N2 N3 N4 N5 N6 N7 N8 gly ala gly gly leu val | _—_ a |
|           |                                |          |
| SigPep1   | ser gly gly ala gly ser ala ser ala leu ala lys ile gly ala gly gly leu val | 0.15 ± 0.06 b |

#### Positive control clone

|         |                               |          |
|---------|-------------------------------|----------|
| SIRIII-8| ser gly gly ala gly leu pro ala ser leu pro ser phe gly ala gly gly leu val | 6.32 ± 1.37 b |
| SIRIII-5| ser gly gly ser gly val pro pro leu phe ala met leu gly ala gly gly leu val | 7.89 ± 1.38 b |
| SIRIII-11| ser gly gly ala gly leu ile ala arg ala val thr ser gly ala gly gly leu val | 7.58 ± 3.72 b |
| SIRIII-30| ser gly gly ala gly pro arg pro thr arg ile ala phe gly ala gly gly leu val | 3.10 ± 0.26 b |
| SIRIII-35| ser gly gly ala gly pro thr arg ala arg val thr ser gly ala gly gly leu val | 0.89 ± 0.17 b |
| SIRIII-11| ser gly gly ala gly leu gly arg arg ala gin leu ser gly ala gly gly leu val | 0.35 ± 0.06 b |
| SIRIII-12| ser gly gly ala gly leu pro pro pro pro leu ala met gly ala gly gly leu val | 0.35 ± 0.10 b |
| SIRIII-1 | ser gly gly ala gly ala his leu ala his pro pro lys gly ala gly gly leu val | 3.12 ± 0.52 c |
| SIVIII-10| ser gly gly ala gly ser gin thr met thr phe ala thr gin gly ala gly gly leu val | 1.82 ± 0.21 f |
| SIVIII-15| ser gly gly ala gly arg arg ala ser ala gin val asn gly ala gly gly pro val | 1.23 ± 0.09 c |
| SIVIII-2| ser gly gly ala gly his pro ser gin glu leu glu ser gly ala gly gly leu val | 0.91 ± 0.16 c |

#### Un-reactive clones

| Clones    | Translated amino acid sequence | Activity |
|-----------|--------------------------------|----------|
| SIRIII-8  | ser gly gly ala gly ser pro tyr arg ile leu leu ala gly gly leu val | NR d |
| SIRIII-56 | ser gly gly ala gly arg his phe ala arg ala asn gly ala gly ser leu val | NR |
| SIVIII-13 | arg ser gly gly ala gly met ala ala pro leu ala asp leu gly ala gly gly leu val | NR |
| SIRIII-37 | ser gly gly ala gly leu val tyr ala arg ser ser pro gly ala gly gly leu val | NR |
| SIRIII-39 | ser gly ser ala gly ala pro ser thr val leu gly ala gly gly leu val pro gin | NR |
| SIVIII-57 | ser gly ser ala gly ala pro leu pro his lys ser pro gly ala gly gly leu val | NR |
| SIVIII-58 | ser gly gly ala gly ile phe arg lys ala arg tyr arg ser gly ala gly leu val | NR |
| SIVIII-2  | ser gly gly ala gly ala pro leu ala pro his lys ser pro gly ala gly gly leu val | NR |
| SIVIII-5  | ser gly gly ala gly arg pro leu pro gin leu pro asp gly gly gly leu val | NR |
| SIVIII-6  | ser gly gly ala gly phe ser tyr met ala met ala ala gly ala gly gly leu val | NR |

*Not applicable.
*Measured with 15 µM SpsB.
*Measured with 17.5 µM SpsB.
*Not reactive. These individual clones were subjected to an activity screen protocol with a ≥2-h incubation with 17.5 µM SpsB as described under “Experimental Procedures.” Hence, those that would show activity with a more extended incubation might still be considered as unreactive.
peptides were 12 residues long (Table II). Three reaction mixtures were set up for each peptide containing 250 μM peptide only (peptide only; see Fig. 3), 17.5 μM SpsB only (SpsB only), or 250 μM peptide and 17.5 μM SpsB (digest). Electrospray ionization mass spectrometry was employed to detect products produced from the proteolysis of peptide by 17.5 μM SpsB following a 16.5-h incubation at 25 °C. Shown in Fig. 3 are the results from an LC/MS experiment in which the digestion of SIRIII-35pep (peptide 5) was analyzed. The incubation resulted in a decrease in the mass signals due to the starting peptide (Fig. 3, SIRIII-30pep), and 15°C. SpsB only (+), SIRIII-30 (●), and SIRIII-35 (△). The lines represent the linear curve fit of data to Equation 2, and the slopes are shown in Table III. A single data point determined for SigPep1 was also included (○).

Reactivity of Synthetic Peptides—The same reaction mixtures used in the LC/MS experiments were also analyzed using HPLC. Upon 16-h incubation of 250 μM peptide with 17.5 μM SpsB, we found that peptides 2, 5, and 6 were cleaved 20, 87, and 62%, respectively (Table III). The cleavage of peptide 3 was complete within 6 h, whereas it took only 4 h for peptide 4, and peptide 1 was completely resistant to cleavage. Based on the extent of cleavage, we determined that peptide 4 was the most active, followed by peptides 3, 5, and 2. The activity of peptides 2 and 4 was further assayed under steady-state conditions using the HPLC method described under “Experimental Procedures.” We found that for both peptides the activity remained linear to substrate concentration in the range tested (Fig. 4). The highest concentration tested for peptide 4 was 500 μM, indicating a $K_m$ of $>500 \mu M$. $k_{cat}/K_m$ was 0.339 and 23.2 $s^{-1}$ for peptides 2 and 4, respectively, calculated from the slope of the linear curve fit to data shown in Fig. 4.

**DISCUSSION**

Having an efficient *in vitro* assay not only can facilitate the biochemical study of an enzyme but is also imperative to inhibitor screens if the enzyme is a potential drug target. For an enzyme such as a protease, which catalyzes transformations to protein substrates under physiologic conditions, finding a peptide substrate is usually the first step toward achieving this goal, and phage display technology has been instrumental to the discovery of substrates for proteases.

**Substrate Phage Reactivity**—We recently described the detailed kinetics for the proteolysis of substrate phage and provided a quantitative basis for the design of a phage library screening experiment (25). We further formulated a screening strategy for meeting the desired outputs through the control of screening stringency. In the present study, we methodically applied the strategy to the signal peptidase SpsB from *S. aureus* and succeeded in finding reactive substrate phage for the enzyme, validating our screening strategy. In the five independent screening experiments performed, the screening stringency was controlled by varying the length of time the phage library was incubated with the enzyme. The incubation was as short as 30 min to as long as 24 h, but only when it was extended to 24 h did we start to uncover a significant number of active clones (Table I). The overall number of active clones was quite small, but it increased as we relaxed the screening stringency, confirming our prediction that a relaxed screening condition would facilitate the enrichment of reactive substrates.
We recognize that adopting such a condition is especially important for an enzyme like signal peptidase, which appears to have a low catalytic efficiency. The single-exponential kinetic process, which we described previously for the reaction of stromelysin with its substrate phage (25), was shown here for the signal peptidase. Furthermore, the reaction of the signal peptidase with substrate phage obeyed pseudo-first-order kinetics (Fig. 2), suggesting that the kinetic model developed using stromelysin (25) should be generally applicable to other enzymatic systems.

Although the criterion we used to define the reactivity of a phage clone was somewhat arbitrary (Table I), the fraction of active clones identified out of the total number of colonies screened was apparently small, implying that only a small population of potential substrates for SpsB was present in the phage display library. The activity of the best clones was not especially high (Table II), and it proved difficult to achieve further enrichment probably due to the low abundance of potential substrates in the library. It is conceivable that the segment displayed on phage needs to be over eight residues.

**TABLE III**

Activity of phage and synthetic peptide substrates of signal peptidase SpsB and the substrate cleavage pattern

| Phage Clone | $k_1$ | Peptide Sequence | Digestion | $k_{cat}/K_m$ |
|-------------|-------|------------------|-----------|---------------|
| SIIHRIII-8  | 212   | Ac–A G L P A S L P S F G A–NH$_2$ | 0         |               |
| SIIHRIII-5  | 87.1  | Ac–A G V P P L F A M L G A–NH$_2$ | 20 0.339 ± 0.008 |
| SIIHRIII-11 | 40.6  | Ac–A G L I A R V T S G A–NH$_2$ | 100 (6 h) ND |
| SIIHRIII-30 | 36.7  | Ac–A G P T A R V T S G A–NH$_2$ | 87 ND |
| SIIHRIII-35 | 34.1  | Ac–A G P T A R V T S G A–NH$_2$ | 62 ND |
| SigPep1     | ND    | Ac–A G S A S A L A K I G A–NH$_2$ |               |

$^a k_1$ is the slope from the linear curve fit to Eq. 2 as shown in Fig. 2B.

$^b$ The amino acids are abbreviated using the single-letter codes. The segment that is present in the variable region in the corresponding clone is underlined. The peptides that can be cleaved by SpsB are aligned along the cleavage site. Various nomenclatures for protease substrate are depicted above the cleavable sequences. The arrow indicates the point of cleavage and the P1 residue in boldface.

$^c$ The extent of digestion was determined using the HPLC method as described under “Experimental Procedures” following the incubation of 250 pM peptide with 17.5 μM SpsB for 16.5 h or as indicated in the parentheses.

$^d$ The parameter was calculated from the slope of the linear curve fit shown in Fig. 4.

$^*$ ND, not determined.

**FIG. 3.** LC/MS analysis of the proteolysis of peptide 5 (SIIHRIII-35pep) by SpsB. A, total ion chromatograms of three reaction mixtures containing the enzyme only (SpsB only), substrate (5 only), and both enzyme and substrate (digest). S indicates the retention time at which the substrate was eluted, and N and C represent the retention times at which products N and C were found, respectively. B, positive ion mass spectra of eluents at the indicated retention time. The mass of each ion species is indicated.

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The catalytic efficiencies of the peptide substrates for SpsB characterized in this study are not fundamentally different from those found for LepB (15). For instance, $K_m$ for the most active peptide 4 (and most likely for peptide 2 as well) is still on the order of millimolar. Previous studies have shown that remarkable improvements in $k_{cat}/K_m$ can be achieved when the peptide is modified so that it forms micelles, which enhance the enzyme and substrate interaction (26); both $k_{cat}$ and $K_m$ values were found to be improved to a level approaching that of a preprotein substrate (16). It would be interesting to incorporate the various SpsB substrate sequences from this study into a micelle-forming peptide or a preprotein construct and to test what effect it would have on the peptide reactivity. One interesting observation with peptide 4 is that only one residue, an Ala at P1, is present on the P side of the substrate sequence. It is not known what is the minimal requirement for the sequence on the P’ side of the substrate molecule. In a previous attempt to define the minimal substrate, it was found that the E. coli enzyme could cleave peptides with a sequence encompassing −2 and +5 or −7 and +2 residues (15). The crystal structure of β-lactam-acylated LepB has been solved (27); however, it is not clear from the structure what kind of interactions would exist in the active site for P’ residues on the substrate sequence. Evidently, more experiments are needed to probe the contribution of P’ residues to support the activity of a substrate like peptide 4.

Conclusions—This study shows that through systematic tuning of the selection stringency in screening phage display libraries, the isolation of highly reactive substrate phage clones for an enzyme like signal peptidase, which appears inefficient catalytically toward simple peptide substrates, can be achieved. It demonstrates the general applicability of the kinetic model of substrate phage proteolysis and the phage display library screening strategy that we described earlier (25). These experiments resulted in the isolation of the first peptide substrates for signal peptidase SpsB from S. aureus and provided the first insight into the recognition of substrate cleavage site by the enzyme.

Acknowledgments—We thank Chianne Chen and Jamie Feng for technical assistance; Matt Smith for construction of the phage display library and control clone SigPep1; Nikhil Shah, Mark Gao, and Bill Fitch for assistance with the LC/MS instrumentation; and Xueying Lin and Jeff Northrop for assistance with the automation.

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