Abstract

WANG, GUANGQUAN. Identification of Peptide Ligands from Solid Phase Combinatorial Peptide Libraries that Bind Staphylococcal Enterotoxin B (SEB). (Under the direction of Dr. Ruben Carbonell.)

Staphylococcal enterotoxin B (SEB) is a primary toxin that causes food poisoning. It also acts as a superantigen that interacts with the major histocompatibility complex class II molecule (MHCII) and T cell receptor (TCR) to activate large amounts of T cells leading to autoimmune diseases. Highly purified SEB is needed in research and can be used as a standard in current detection methods for SEB. Inexpensive, robust ligands with high affinity would be suitable replacements for antibodies in biosensors for the detection of SEB. Affinity adsorption processes using short peptides as ligands show great promise in purifying and detecting proteins in comparison with other methods due to their high stability and low cost. We have found an affinity peptide ligand, YYWLHH, from a solid phase combinatorial peptide library that can separate SEB from *E. coli* lysate or BSA. Such a peptide ligand is a good candidate to replace antibodies in purification and to develop new biosensors to detect SEB. In addition, the derived peptide ligand might help in understanding the interaction between SEB and MHCII and TCR.

IDENTIFICATION OF PEPTIDE LIGANDS FROM SOLID PHASE COMBINATORIAL PEPTIDE LIBRARIES THAT BIND STAPHYLOCOCCAL ENTEROTOXIN B (SEB)

By

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Biography

Guangquan Wang was born on April 20, 1972 in Boxing, Shandong Province, China, where he attended elementary and high school. He went to Dalian University of Technology in 1990 and obtained his B. S. degree in Chemical Engineering in 1994. Guangquan began his graduate studies at the Chinese Academy of Sciences in August 1994. After one-year of course work at the Graduate School of the Chinese Academy of Sciences in Beijing, he spent two years at the Institute of Chemical Physics at Dalian, Chinese Academy of Sciences, to finish his M. S. in Chemical Engineering. He then worked in the same institute as a research assistant for nearly two years.

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Introduction

Motivation

Staphylococcal enterotoxins (SEs) comprise a family of ten serologically distinct toxins (labeled A-K), all of which are secreted by *Staphylococcus aureus* and share significant homology in primary structure. These toxins are primary causes of food poisoning (Bergdoll, 1983). In addition, they act as superantigens to stimulate much more T cells than conventional antigens do to enhance cytokine release (Swaminathan et al., 1992; Marrack and Kappler, 1990; Johnson et al., 1991).

Due to the limited amount of SEs for intoxication (for example, ~5-20μg/Monkey), it is necessary to develop a sensitive and convenient analytical method to detect such toxins. Furthermore, SEs contaminate other proteins produced from Staphylococcal bacteria, such as protein A. Protein A plays an important role in purification and therapeutic removal of IgG and IgG-containing immune complexes in the treatment of certain cancers and autoimmune diseases (Balint et al., 1989). Thus an extra step is needed to remove the trace SEs and reduce loss of activity and yield of protein A. Homogeneous and highly purified preparations of SEs are required in studies of the biological activity of SEs and the methods of identification and quantification of SEs (Bhatti et al., 1994; Lopes et al., 1996). Hence an efficient and convenient purification method is required to recover enterotoxins from fermentation broth and cell culture. Affinity chromatography is used in laboratory and industrial scales to recover a wide variety of biological molecules, especially when the concentration of product of interest is low and when it is found in solution with many contaminants with similar

physical properties. Thus affinity chromatography is a promising method to detect, remove or purify SEs.

The key in affinity chromatography is to create or find a proper adsorbent. Ideally, a ligand should be specific, inexpensive, and stable during multiple operational cycles. To recover the biological molecules while retaining their activity, the interaction between ligands and target molecules should be moderate so that harsh elution conditions can be avoided (Chase, 1983; Sproule et al., 2000). The primary affinity ligands used in practice are immobilized metal atoms, dyes and polyclonal and monoclonal antibodies. Both metal and dye ligands are pseudo-affinity ligands. These ligands tend to have lower specificity than antibodies. In addition, dyes and metals have the potential to leach from the column to contaminate the products. Monoclonal antibodies have been widely used in protein purification because of their high affinity and specificity. However, monoclonal antibodies must be purified extensively prior to use as affinity ligands. In addition, immobilized antibodies are sensitive to operating conditions. Harsh conditions are usually required to break antigen-antibody interactions in the elution step and even harsher conditions are necessary (ex. 0.1M sodium hydroxide) to clean and generate the columns prior to the next operating cycle. These conditions can denature the antibodies over time so that only limited operating cycles can be carried out. Both the difficult purification and relatively short life of antibodies result in high cost of antibodies as affinity ligands. Furthermore, the leakage of the antibody into the product could elicit an immune response (Huang and Carbonell, 1995; Bastek et al., 2000). As a result, low cost, more robust ligands that have moderate interaction with the target could have widespread potential application. Short peptides derived from combinatorial peptide libraries have

been demonstrated to be good candidates as ligands in affinity chromatography (Lam et al., 1991; Baumbach and Hammond, 1992; Huang and Carbonell, 1995).

SEB is the most widely studied member of the SEs (Swaminathan et al., 1992). It is a good model of a toxin that can be used in biological warfare and other environmental problems. We use SEB as target molecule to find affinity ligands from a solid phase combinatorial peptide library, and then use such ligands to detect, remove and purify SEB by affinity chromatography. In addition, the derived peptide ligands can help us understand the interactions of SEB as a superantigen with the major histocompatibility complex class II molecule (MHCII) and T cell receptor (TCR). As far as we are aware, this is the first time an attempt has been made to identify affinity ligands for a toxin using solid phase combinatorial peptide libraries.

Staphylococcal enterotoxin B (SEB)

Staphylococcal enterotoxins (SEs)

Staphylococcal enterotoxins (SEs) are a family of structurally related proteins that are produced by *Staphylococcus aureus* (Bergdoll, 1983). These toxins function both as potent gastrointestinal toxins to cause toxic shock-like syndromes as well as superantigens that stimulates non-specific T-cell proliferation to result in several allergic and autoimmune diseases (Balaban and Rasooly, 2000). The SEs are single-chain simple proteins (25-30kDa) which contain one disulfide loop in their molecule and are resistant to temperature and pH denaturation (Bergdoll, 1983). To date ten SEs, designated as SEA (Casman et al., 1963), SEB (Casman et al., 1963), SEC (Bergdoll et al., 1965; Reiser et al., 1984), SED (Casman et al., 1967), SEE (Bergdoll et al., 1971), SEG (Munson et al.,

1998), SEH (Su and Wong, 1995), SEI (Munson et al., 1998), SEJ (Zhang et al., 1998) and SEK (Orwin et al., 2001), have been identified and differentiated on the basis of their reactions with specific antibodies. SEC is further divided into SEC1, SEC2 and SEC3 due to minor epitope variations. All these toxins share significant sequence homology. Comparison of the sequences of the SEs indicates that SEA, SEE, and SED form one group with an overall amino acid homology of 51%-81%, whereas SEB and SECs form another group of toxins with sequence homology of 42%-67%. The homology between the two groups is around 22-33% (Marrack and Kappler, 1990; Balaban and Rasooly, 2000). Among recently characterized SEs, SEJ has 52%-66% homology with the first group, but SEI and SEH are more distantly related from both groups. Another enterotoxin produced from *Staphylococcus aureus*, originally designated as SEF, has a distant relationship with other SEs and now has been renamed as the Toxic Shock Syndrome Toxin-1 (TSST1) (Bergdoll, 1985). Its 3D structure is remarkably similar to SEB (Kim et al., 1994). All these toxins have similar mechanisms of pathogenesis.

As gastrointestinal toxins, SEs are the second most common cause of reported food-borne illnesses. They usually contaminate some dairy food products, such as meat, poultry, fish, milk and their products (Swaminathan et al., 1996; Balaban and Rasooly, 2000). Patients with SE poisoning may present with emesis, diarrhea, nausea, dizziness and prostration. Some tests done on monkeys show that the amount necessary to cause intoxication is very small, on the range of ~5-20μg/animal (Bergdoll, 1967). Little is known about the mechanism of this intoxication at present. It is possible that emesis occurs through stimulation of neural receptors in the abdomen by the toxins (Bayliss, 1940).

SEs are well understood to be superantigens. Superantigens cause immense T cell activation that can be several orders of magnitude greater than that evoked by routine antigens. This is due to their different modes of interaction with the major histocompatibility complex class II molecule (MHCII) and T cell receptor (TCR) from those of a routine antigen. Routine antigens have to be preprocessed by the antigen-presenting cells (APC) into small peptides (13 to 17 residues in length). These antigenic peptides bind to a cleft formed by two α helices of the α and β chains of the MHCII molecule. The formed peptide-MHCII complex makes contact with all variable chains of TCR and the fraction of T cells stimulated is limited by these interactions (Goldsby et al., 2000; Swaminathan et al., 1992). Usually only 0.0001%~0.001% of T cells get activated this way (Marrack and Kappler, 1987). Superantigens need no such preprocessing. They bind as intact molecules but to a different part of the MHCII molecule than normal antigenic peptides. The superantigen-MHCII complexes make contact only with the Vβ chain of TCR. Different SEs have distinguishable specificities to TCR and MHC II even though they fall into the same group based on sequence homology (Swaminathan et al., 1996). The formation of MHCII-SE-TCR ternary complexes triggers the proliferation of all T cells bearing particular types of Vβ elements. Since there are only a limited number of Vβ elements in humans, a very large fraction (2~20%) of T cells get stimulated. The consequent release of T cell-derived lymphokines such as interleukin-2 or tumor necrosis factor may be involved in the mechanism of toxicity, causing fever, weight loss, and osmotic imbalances that could lead to death (Swaminathan et al., 1992; Marrack and Kappler, 1990; Johnson et al., 1991). Although it is well known that the emetic function

is dissociable from that of T cell stimulation, both emesis and T cell stimulation are dependent upon distinct but overlapping regions on SEs (Harris et al., 1993).

Characteristics of SEB

SEB is a monomer protein with a MW 28,366 Daltons and pI 8.6. The three-dimensional structure of SEB determined by X-ray crystallography reveals a molecule containing two domains composed of residues 1-120 and 127-239 respectively (Figure 1) (Swaminathan et al., 1996; Papageorgiou et al., 1998). Domain 1 consists of two β sheets, one formed by β1, β4 and β5 and the other by β2, β3, β4 and β5. It also contains three α-helices, α1, α2 and α3. Domain 2 mainly consists of two α helices, α4 and α5 and a twisted β sheet formed by β6, β7, β9, β10 and β12; it also includes two very short β strands, β7 and β11. SEB binds to MHCII with high affinity ($K_d \sim 10^{-6}$) (Scholl, et al., 1989) and to T cells bearing Vβ3, 7, 8.1, 8.2, 8.3 and 17 in mice (White et al., 1989). Based on the topology and mutational studies of SEB, the T-cell receptor-binding site on SEB encompasses a shallow cavity formed by domains 1 and 2. The MHCII molecule binds to an adjacent site, namely the α5 face of the SEB molecule. The active site for emesis is thought to be in the α4 groove (Swaminathan et al., 1992).

SEB is one of the most heat-stable proteins. The activity loss due to heat is fast initially and then levels off. Less than 50% activity is lost when the toxin is heated to 100°C at pH 7.3 for 5 mins (Schantz et al., 1965). Heat aggregation results in rapid loss of activity at 70 to 80°C. But heating to 100°C can recover 35 to 40% immunological activity because of the dissociation of aggregates at higher temperature. Therefore, heat treatment of SEB causes a more rapid loss of immunological activity at 70 to 80°C than at 90 to 100°C. After incubation at 25°C, some activity of toxins that have been

inactivated by heat can be recovered (Satterlee et al., 1969; Jamlang et al., 1971; Fung, et al., 1973).

SEB is resistant to denaturation when it is exposed to denaturants, such as guanidine hydrochloride and urea. Isothermal denaturation experiments show that prolonged exposure (hours to days, depending on denaturant conditions) of SEB in guanidine hydrochloride up to 6M and urea up to 9M is required for unfolding to reach equilibrium. Refolding the denatured toxin to native protein after dilution of denaturant is complete within minutes to a few hours. This indicates a very large activation energy for unfolding and a comparatively small activation energy for refolding of SEB. The stable structure of SEB is due to the disulfide loop that locks covalently two β sheets in a stable, anti-parallel configuration (Warren, 1974, 1977). However, SEB can be denatured rapidly and irreversibly when the pH is below 3.5. This is because SEB is a basic protein, and hence low pH results in protonation of carboxylate groups that are involved in maintaining the native SEB conformation. Such protonation leads to a conformational change of SEB so that the local positive electrostatic free energy resulting from protonation of the carboxylate groups can be reduced (Warren et al., 1974).

It is a challenge to deal with microheterogeneity of SEB when characterizing SEB. Different preparation and purification methods give different SEB-associated forms. It is generally agreed that only one component is synthesized within *Staphylococcus aureus* strains. The hydrolysis of labile amide groups of glutamine and asparagine residues in the protein converts the original component to less alkaline forms of SEB by amidohydrolase in the fermentation broth. Thus there are series of isomers of SEB that are different in pI, but serologically identical. This has been confirmed by the

comparison between the amino acid sequence deduced from the DNA sequence, and the amino acid sequence analyzed from purified SEB by Edman degradation, where most of the differences involve aspartic acid and asparagines, and glutamic acid and glutamine (Huang and Bergdoll, 1970; Jones and Khan, 1986). The distribution of the multiply-charged SEB species is dependent on the operation conditions, especially temperature and pH, in the preparation methods employed (Baird-Parker and Joseph, 1964; Schantz et al., 1965; Chang and Dickie, 1970; Metzger et al., 1971; Spero et al., 1974). In addition, polymerization of SEB usually develops during storage, and low ionic strength can enhance that process (Jamlang et al., 1971).

Detection of SEB

The required detection level of enterotoxin in foods from outbreaks is 0.1~0.2μg/100g of food (Bergdoll, 1979). Most of the methods for detection of enterotoxins are based on the use of antibodies prepared against the enterotoxins. One of them is the gel diffusion method. Polyclonal antibodies prepared in rabbits using the individual purified enterotoxins react with the enterotoxins in gels to give precipitin reactions that are highly specific (Bergdoll, 1996). The used gels are generally Ouchterlony gel plates and microslides. The normal sensitivity of gel diffusion methods is around 0.1~0.5 μg/ml (Robbins et al., 1974; Meyer and Palmieri, 1980). The reversed passive latex agglutination method (RPLA) is more sensitive than gel diffusion methods. The antibody-coated latex particles agglutinate when brought in contact with the enterotoxins. RPLA is sensitive enough to detect enterotoxins in most foods that cause food poisoning (Weineke and Gilbert, 1987). However, enzyme-linked immunosorbent assay (ELISA) are usually employed instead of RPLA to detect small amounts of

enterotoxins. Several kits are available commercially (Fey et al., 1984; Park et al., 1994). The antibody is treated with the sample and then the antibody-enterotoxin complex is incubated with the enzyme-antibody conjugate. The color developed from enzyme-substrate reaction is directly proportional to the amount of enterotoxin in the sample. Thus ELISA can be used directly on crude extracts from food or partially purified samples. Most of the sorbents used to attach antibodies are microtiter plates, others include polystyrene spheres, tubes, and dip sticks with wells. The sensitivity of an ELISA is usually less than 1 ng/g food (Fey et al., 1984; Bergdoll, 1996). However, occasional antibody cross-reactivity with unrelated antigens and the insensitivity with heat-treated foods prevent definitive identification of the enterotoxin with the use of an ELISA (Park, et al., 1992; Rasooly and Rasooly, 1998). Western blotting can overcome these major problems because the Western procedure solubilizes denatured toxin, which may still be biological active, and allows characterization of antigen that reacts with the antibody (Rasooly and Rasooly, 1998). The most sensitive detection method (at pg level) is the T-cell proliferation assay which measures the ability of enterotoxins to act as superantigen. However, this assay is nonspecific since any enterotoxin in the sample can induce T-cell proliferation. Thus one more step such as an ELISA is subsequently needed to identify the enterotoxin (Rasooly et al., 1997). All these immunological methods share the same disadvantage. They are relatively time-consuming, ranging from several hours up to several days.

Biosensors based on immunological testing are an ideal alternative to the immunological methods mentioned above. The limits of detection and sensitivity of biosensors are comparable to ELISA, but the total assay time could be only a few

minutes. The detection mechanism of biosensors is based on the interaction between a surface-immobilized receptor, mostly an antibody, and a solution-born analyte. The consequential biological response of the antigen-antibody interaction is then translated into an electronic output that can be analyzed (Rasooly and Rasooly, 1999; Nedelkov et al., 2000). There are two common detection methods used in biosensors. One is based on surface evanescent waves, which can be used to measure the changes in refractive index due to antigen-antibody interactions at the surface of optical waveguides; these changes can be recorded as a shift in resonance angle (Rasooly and Rasooly, 1999). The other is based on surface plasmon resonance (SPR), where the change in the refractive index at the surface results in a measured shift in the wavelength of light absorbed, or by light diffraction (Nedelkov et al., 2000). The major problems of ELISAs, such as antibody cross-reactivity with unrelated antigens and the insensitivity with heat-treated foods, can also occur with biosensors. As in ELISA, the use of sandwich format immunoassay that involves a secondary antibody could increase the specificity in a biosensor. A fiber-optic biosensor system that includes a secondary fluorescent-labeled antibody can be used to detect SEB with little cross-reactivity to SEA and SED (Tempelman et al., 1996; King et al., 1999). Recently, biomolecular interaction analysis mass spectrometry (BIA-MS) has been developed to differentiate specific antibody-enterotoxin binding from nonspecific antibody-antigen binding in SEB detection, where a second step, matrix-assisted laser desorption /ionization time-of-flight mass spectrometry, is used to identify SEB (Nedelkov et al., 2000). These specific biosensor systems require complex instruments and are not as convenient as ELISAs.

Purification of SEB

The determination of the biological activity of SEs requires highly purified and homogeneous enterotoxins. Meanwhile, the methods of identification and quantification of SEs, such as ELISA and biosensors, also require highly purified enterotoxins as standards. Thus it is necessary to develop corresponding methods for the purification of SEs. SEs are produced within *Staphylococcus aureus* strains and are released in the fermentation growth medium. SEA, SED and SEE belonging to the same group based on sequence homology are synthesized and released into the growth medium near the beginning of the exponential growth phase, whereas SEB and SECs belonging to another group are produced during the late stationary phase (Baird-Parker, 1971; Spero et al., 1987). *Staphylococcus aureus* strains, which release one dominant enterotoxin, and the corresponding growth conditions, are known for most of the SEs. With the use of a highly SEB-productive strain, *Staphylococcus aureus* S-6, the concentration of SEB can reach 270μg per ml culture supernant after 48-h fermentation (Donnelly et al., 1970; Lopes et al., 1996).

Challenges presented in the purification of SEs from fermentation broth include: 1) contaminants with similar molecular weights and charge to the enterotoxin of interest, such as other enterotoxins, protein A, proteases, staphylokinase, hemolysins or endotoxin (Bhatti, et al., 1994); 2) microheterogeneity of SEs, which results from polymerization or hydrolysis of labile amide groups. Different techniques have been used to purify SEs. Most of these are developed from the purification of SEB. Only minor variants are needed to apply these techniques to purify other enterotoxins because all SEs share a great structure homology and have similar properties. The degree of purity and percent recovery are the major criteria for chracterizing SEB purification methods. Most of the

methods developed to date seldom guarantee both (Table 1). Although multi-step procedures give high purity, they usually give low yield of SEB and take a long time, and cannot easily be adapted for large-scale purification. Chromatography methods give high recovery and purity compared to chemical methods, such as acid or ethanol precipitation (Table 1). Different chromatography methods, such as cation exchange, hydrophobic, reverse-phase or chromatofocusing chromatography, have been used successfully to recover SEB in large-scale. However, these methods are not specific enough to get highly purified and homogeneous SEB. Additional steps, such as gel filtration, electrophoresis or isoelectric focusing, are needed to polish the partially purified enterotoxin (Table 1). Dye ligand affinity chromatography is a kind of psudo-affinity chromatography method, which is more specific than the chromatography methods mentioned above. The adsorption of enterotoxins to dye ligands results from a mixture of interactions consisting of electrostatic, hydrophobic and hydrogen bond interactions. Large-scale purification of SEA, SEB and SEC_2 with one-step dye ligand chromatography by using Red A as ligand has been demonstrated by Brehm et al (1990). But there are still some contaminants in the preparation of SEB after dye ligand chromatography. A pre-purification step, such as cation exchange, is recommended to remove a portion of the contaminants prior to Red A chromatography (Lopes, et al., 1996). Thus more specific ligands are needed to capture SEB in one-step so that better recovery and purity can be obtained.

Affinity ligands from combinatorial peptide libraries

Advantages of peptide ligands

Affinity chromatography is the most efficient way to purify biomolecules. Many biomolecules can be purified within one step using affinity chromatography with high recovery. The key in affinity chromatography is to find a proper affinity ligand corresponding to the molecules of interest. While monoclonal antibodies are the most common ligands for laboratory uses, it is difficult to scale up these columns because antibodies are quite costly and sensitive to operating conditions. Also, the leakage of the antibodies under harsh elution and cleaning conditions can result in serous contamination due to the immunogenicity of these proteins. Usually affinity chromatography with monoclonal antibodies as ligands is used to polish the product in the last step of purification. Dye ligands and immobilized metal ions are more stable than antibodies so that they can stand harsh operation conditions, but both of them are lack of sufficient specificity. In addition, dye ligands are toxic and there is severe leakage of immobilized metal ions. Prior experiences using peptides as ligands ranging from 3 to 25 amino acids has shown that peptides have affinities to molecules of interest that compare well with dyes and immobilized metal ions. As opposed to monoclonal antibodies, small peptide ligands are much more stable because they don't require a specific tertiary structure to maintain their biological activity. Small peptides are also not likely to cause immune response in case of leakage into the products and it is easy to isolate peptides from the products downstream. Peptides can be manufactured aseptically in large scale under GMP (good manufacturing practices) conditions at relatively low cost. The interactions between peptides and proteins are moderate so that the protein can be eluted under mild

conditions without damage to protein activity (Baumbach and Hammond, 1992; Huang and Carbonell, 1995; Huang et al., 1996; Huang and Carbonell, 1999; Bastek et al., 2000). In addition to being good candidate as ligands in affinity chromatography, peptides are used widely to determine protein-protein interactions without a priori information on protein structure (for example in epitope mapping).

Combinatorial libraries for peptide ligand detection

One of the challenges for use of peptide ligands is the identification of a sequence that shows affinity and specificity to the target protein. The design of specific complementary peptide sequence to the target protein has been demonstrated difficult even when the structure of the target protein is known (Lawrence and Davis, 1992; Saragovi et al., 1992). The development of combinatorial libraries has allowed screening millions of peptide sequences to discover specific peptides that bind to the target protein. Peptide libraries can be generated either biologically or synthetically. Several combinatorial library methods have been described in the literature (Lam et al., 1997). The most widely used biological libraries are phage-displayed libraries, while one-bead-one-peptide libraries are the dominant libraries obtained directly from chemical synthesis.

Phage-displayed random peptide libraries

In phage-displayed peptide libraries, a random gene with a given length is synthesized and then inserted into bacterial phage gene III. The corresponding peptide coded by the inserted DNA is displayed at the N-terminal of the gene III protein (pIII) on the phage surface. Each phage displays one kind of peptide sequence that is different from other phages. Affinity peptides on phage that bind to the target protein are selected through several rounds of affinity purification. Namely, millions of phage particles are

incubated with the target protein that has already been immobilized on a Petri dish or ELISA plates. Non-binding phages are washed out extensively, and then the bound phages are eluted under harsh conditions. The eluted phages are then amplified on agar medium and subjected to the next round of affinity purification. The tight-binding phages are then cloned and propagated in *Escherichia coli.* The amino acid sequence of the peptide on the phage is deduced by sequencing the coded DNA in the phage gene III (Scott and Smith, 1990; Devlin et al., 1990; Cwirla et al., 1990).

Ligands identified from phage libraries frequently interact with natural binding sites on the target molecule and resemble the target's natural ligands. Thus phage-displayed random peptide libraries have been used to investigate protein-protein interactions in a variety of contexts. For example, phage-displayed random peptide libraries have been used to map the epitopes of monoclonal and polyclonal antibodies, and to identify peptide ligands for receptors, receptor ligands, and folded domains within larger proteins, such as several SH2, SH3 domains (Daniels and Lane, 1996; Zwick et al., 1998). Recently, peptide ligands for some superantigens, for example, SEB and TSST-1, have been determined with phage-displayed random peptide libraries (Sato et al, 1996; Goldman et al., 2000). But biopanning with phage-displayed libraries is slow and subjected to non-specific binding.

The most important aspects in the determination of peptide ligands using phage display are the construction and maintenance of libraries with sufficient structural diversity and the efficient selection of specific binding phage from nonspecific binding phage. Phage-displayed random peptide libraries have been constructed to display peptides of variable length ranging from 6 to 38 amino acids (Daniels and Lane, 1996).

Once it is created, a phage library can be regenerated continuously and re-used unlike a synthetic library. The problem in phage library construction is that the library may not be truly random due to genetic bias in the creation of these libraries. The efficiency of screening can be controlled by adjusting the washing conditions in the screening process (D'Mello and Howard, 2001). But it is also possible that the specific peptide on phage that is finally selected is not the original one from the library because of some biological bias in amplification and propagation of the phage. Care must be taken to maintain the diversity of the libraries. Usually the diversity of the original phage library is on the order of 10^8 peptides. Selection must be avoided during the expansion and propagation of library for phage with selective growth advantage (Daniels and Lane, 1996). In addition, the resources of the peptide synthesis on phage are limited to the 20 natural amino acids so that D-amino acids or other molecules cannot be used to increase the diversity of the library.

One-bead-one-peptide libraries

Synthesized libraries are created on solid supports through organic chemistry. There are several distinct combinatorial library methods (Lam et al., 1997). The one-bead-one-peptide library method is used extensively in drug discovery processes due to its unique features (Lebl et al., 1995). Compared to other methods, the synthesis of a one-bead-one-peptide library is rapid with use of the "split synthesis" approach. Because one bead has one unique peptide sequence, all of the beads can be tested concurrently but independently. Once positive beads have been identified, the chemical structure of the peptides on the beads can be directly determined by sequencing or by an encoding strategy. In addition, the libraries can be used either in the solid phase (i.e. peptides

attached on solid) or solution phase (i.e. peptide cleaved from solid support). As in phage-displayed libraries, the screening of peptide ligands from one-bead-one-peptide libraries involves three steps (Lam et al., 1997): (i) construction of the library, (ii) screening the library with the target molecule, (iii) determination of the peptide sequence. We will give a brief discussion of the application of one-bead-one-peptide libraries in determining peptide ligands specific for a biomolecule of interest.

Construction of the one-bead-one-peptide libraries

The first one-bead-one–peptide library was synthesized by Lam et al. (1991) using the "split synthesis" approach developed by Furka et al. (1991)(Figure 2). The resin beads are divided equally into separate reaction vessels each with a single amino acid. After the first amino acid is coupled to the resins, beads are repooled, mixed thoroughly, and redistributed into separate reaction vessels. The next coupling step is then performed. This divide-couple-recombine technique is repeated until the desired length of the peptide library is reached. There are X^n random sequences in the library, where X is the number of amino acids used for coupling, and n is the length of the library. Each resin bead displays only one peptide sequence. Thus libraries of this type are called "one-bead-one-peptide" libraries (Lam et al., 1991). Because other ligands besides naturally occurring amino acids, such as D-amino acids, oligonucleotides, synthetic oligomers, proteins and small molecules, also can be coupled to solid resins, the idea of a one-bead-one-peptide library has been extended to one-bead-one-compound library (Lam et al., 1997). The introduction of other compounds besides amino acids in combinatorial library construction increases the diversity of the library in comparison with a phage-displayed peptide library, in which phages only display peptides composed of natural amino acids.

However, all synthetic methods have a practical limit on the size of the library as well as the length of the peptides on beads, while peptides on phage can be fairly large.

The choice of the solid support is critical for the library construction and the following application of the library. The biological signal released from the peptides on a single bead quantitatively depends on the amount of the peptide on the bead. As a result, the size and substitution homogeneity is of the utmost importance. Meanwhile, the resin should resist the formation of clusters because cluster would prevent the statistical distribution of resin beads and lower the number of structures created. In addition, resins should be compatible with various organic and aqueous media. Solid beads with porous structure are preferred. The high surface area that porous resins provide can attach more ligands, facilitating bead sequencing and providing high capacity for their usage in chromatography. Moreover, the pores should be large enough to eliminate diffusion resistance especially when using large proteins as targets. In order to avoid nonspecific binding between the solid matrices and proteins, hydrophilic resins are preferred. If the peptide ligands will be used to purify protein in chromatography, the resins should have enough mechanical rigidity to withstand the high pressure used in liquid chromatography. A variety of polymer beads have been used to attach peptides in library construction, including polyhydroxylated methacrylate, polydimethylacrylamide, polyoxyethylene-grafted polystyrene, TentaGel and so on (Buettner et al., 1996; Lam et al., 1997).

Screening of the one-bead-one-peptide libraries

The identification of the specific peptide ligands to the target protein from a one-bead-one-peptide library depends on the screening methods used. Both solid-phase and solution phase methods have been developed for the one-bead-one-peptide combinatorial

library method. The most widely adopted method of screening is the "on-bead" binding assay (Lam and Lebl, 1994, Lam et al., 1997). The target protein is incubated with the library beads. The library beads with specific peptide sequence to the target protein bind the target protein. The binding of the target to the bead-bound ligands is usually detected by using a reporter group such as an enzyme, a radionuclide, a fluorescent probe, or a color dye covalently attached to the target molecules. Alternatively, antibodies can also be used in the detection scheme as in ELISA. The signals generated from these reporter groups are proportional to the amount and density of peptides on the bead. Nonspecific binding can result in high background resulting in some difficulty in determining the affinity ligands. This usually is eliminated by using a high ionic strength buffer (e.g. 0.2-0.4M NaCl) to reduce purely electrostatic binding and blocking proteins (e.g. casein or bovine serum albumin) and nonionic detergents (e.g. 0.1% Tween 20).

One of the most convenient screening methods is the enzyme-linked colorimetric detection scheme. It has been used to discover the binding motifs for streptavidin (Lam et al., 1991; Lam and Lebl, 1992), avidin (Lam and Lebl, 1992), monoclonal antibodies (Lam et al., 1996), proteases (Lam et al., 1996), and MHC molecules (Smith et al., 1994). The alkaline phosphatase coupled target protein is used to bind to library beads, and then the substrate of alkaline phosphatase, 5-bromo-4-chloro-3-indolyl-phosphate (BCIP) is added. The reacting beads turn turquoise, while the majority of the beads in the library remain colorless. The positive turquoise beads are isolated and then sequenced by Edman degradation. The enzyme-linked colorimetric detection method is extremely rapid, taking a few hours to screen 10^7-10^8 beads. The problem with this method is that the enzyme molecule attached to the target can sterically affect the binding of the target to peptides

on beads. Radionuclide-labeled targets can be used to screen library beads to avoid this problem. The radionuclide probes, such as ^{3}H and ^{14}C, are particularly small compared to enzyme as reporter groups on the target, and it has been demonstrated that the labeled target shows almost same biological properties as the natural target. The library is incubated with the radiolabeled target protein, washed, and then suspended in agarose gel. The slurry is poured onto a gel bond to form a monolayer so that all beads are spatially separated. Exposure of the gel to autoradiography film can locate the positive beads that are then isolated and sequenced. Several researchers have screened peptide libraries using radiolabeled targets (Kassarjianet al., 1993; Turck, 1994; Nestler, et al., 1996; Mondorf et al., 1998). The method developed by Mondorf et al. (1998) using ^{14}C offers high resolution and sensitivity. It has been used to identify affinity peptide ligands for s-protein (Mondorf et al., 1998), fibringen (Mondorf et al., 1998), Alpha-1-proteinase inhibitor (Bastek et al., 2000), α-lactalbumin (Gurgel et al., 2001) and recombinant factor VIII (Chen et al., 2000). Immunostaining schemes similar to ELISA also can be used to target the protein on beads. There are no modifications of the target using this method, so the bead-bound ligands bind directly to the native protein, and not to any adducts. However, the antibodies used in the detection system could bind to bead-bound ligands besides the targets to bring the possibilities of interference and false positives. A two color PEptide Library Immunostaining Chromatographic ANanlysis (PELICAN) has been developed to determine beads specific for the target from those beads resulting from antibody cross reactivity (Buettner et al., 1996). Peptide ligands used to purify protease factor IX and fibrinogen were identified with the use of the PELICAN method (Buettner et al., 1996; Buettner et al., 1997). Other on-bead screening schemes try to use dye-

labeled targets or fluorescently labeled target (Chen et al., 1993; Needels et al., 1993). However, dyes always complicate the screening process by binding to many peptide ligands directly, while autofluoresence of the library can make the library unsuitable for this kind of screening process (Lam et al., 1997). In order to minimize the number of false positive beads, two orthogonal screening methods can be used for one target. For example, a dual-color detection scheme (Lam et al., 1995) and a cross-screening scheme with enzyme-linked colorimetric and radiolabeled assays (Liu and Lam, 2000) have been developed. In this way, many of the initially determined positive beads are eliminated, and the chances to get the true positive beads are greatly enhanced.

One of the disadvantages of on-bead screening is the high peptide density required for peptide sequencing on beads. This can lead to multiple-point attachment of the target to the peptides so that nonspecific interaction between target and peptides will be enhanced. Thus the selected peptide ligands may have less affinity and specificity to the target. Screening of soluble peptide libraries can make the affinity ligands more selective. The format of affinity chromatographic screening developed by Evans et al. and Huang & Carbonell is suitable for screening peptide libraries due to its rapidity (Evans et al., 1996; Huang and Carbonell, 1999). The targets are immobilized onto resins and then packed into a chromatographic column. The soluble peptide libraries are pumped into the column at a proper flow rate to ensure the peptides have enough time to bind to the immobilized targets. Then the column is washed thoroughly with binding buffer. The affinity peptide ligands bound to the targets are eluted and isolated by reverse-phase chromatography. The fractions are then sequenced by Edman degradation or mass spectrometry. Huang and Carbonell have demonstrated this technique by

showing that the consensus sequence, NFVE that binds to the s-protein, is the same as that found from the screening a phage displayed library (Huang and Carbonell, 1999). Evans et al. used a similar system to recover the known epitope, YGGFL, for monoclonal antibody (3E-7), and then determining the affinity ligands for bacterial lipopolysaccharide (LPS, endotoxin) (Evans et al., 1996). Although this technique is rapid and able to avoid false signals from nonspecific binding, it misses some affinity peptide leads that generally cannot be missed by using on-bead screening. The kinetics of the chromatography and the orientation of the immobilized targets, may limit the contact between the immobilized targets and free peptide ligands. In addition, the methodologies used in this technique are more complex than those in on-bead screening (Huang and Carbonell, 1999).

Protein purification by peptide ligands

Although peptide ligands from phage library have been presented on chromatographic support to purify proteins (Baumbach and Hammond, 1992; Huang and Carbonell, 1995; Huang et al., 1996), it is possible that the microenvironment and the orientation of the peptides on the chromatographic support could be very different from that on phage. This can affect adversely the interactions between the peptide ligands and the target (Buettner et al., 1996). It has been found that some peptides derived from phage library only work when the peptide is an integral part of the phage coat protein and not when isolated in free solution (Lowe, 2001). Chemically derived peptides are synthesized and screened on solid beads. They can be used directly to purify protein by chromatography. Thus one-bead-one-peptide libraries are widely used to discover peptide ligands for protein purification.

The affinity and specificity of the peptide ligands from the screening of the peptide library can be confirmed and further demonstrated using a chromatography format. Some peptide ligands identified by library screening are bioselective to their targets, while other peptide ligands are pseudo-affinity ligands behaving like dye ligands. The study by Huang et al. shows that the peptide ligand, YNFEVL, is so specific to s-protein that randomization of the peptide sequence destroys the binding (Huang and Carbonell, 1995). A similar study on the binding of von Willebrand Factor to the peptide RLRSFY shows that the randomization has little effect on the binding (Huang et al., 1996). The pseudo-affinity peptide ligands are better used to capture the target at the first step in purification (Bastek et al., 2000); other steps such as filtration are needed to polish the products. The bioselective peptide ligands can be efficient for purifying the protein in one step, but usually preparation prior to the affinity chromatography step is needed to protect the ligands and maximize the efficiency of the affinity column. Basically, the affinity of the selected peptide ligands depends on the screening of the peptide library. Based on the behavior of the peptide ligands in purifying target in a column format, it is possible to adjust the screening conditions to yield proper ligands for purification.

Ligand density can affect the interactions between the peptide ligands and the target protein. If the binding is attributed to monovalent interactions, the capacity increases when increasing the ligand density, while the association constant may remain constant at low ligand density and decrease at high ligand density due to steric effects. Thus there is an optimal density at which the peptide ligands have high capacity and an acceptable extent of steric hindrance. If the binding is attributed to multivalent interactions, increasing the ligand density typically increases the capacity and association

constant. For highly specific ligands, increasing the ligand density may increase the steric hindrance at the surface and make the binding less efficient, namely, decreasing the association constant and the utilization of the ligands. Small protein molecules are expected to have monovalent interactions with the adsorbent. The binding of s-protein to a peptide sequence, YNFEVL, has been shown to be 1:1 specific (Smith et al., 1993). Further adsorption isotherm measurements in a batch system have shown that the binding capacity increases from 0.0466μmol/ml to 1.1650μmol/ml, while the peptide utilization decreases from 96% to 40%, and the binding constant decreases from 1.2×10^5 to 5.6×10^4 M^{-1} when the peptide density increases from 0.05 to 3.0μmol/ml (Huang and Carbonell, 1995). It is more likely that the binding of large protein molecules to peptide ligands is multivalent because there may have several interaction sites at their surfaces. The results of binding of von Willebrand Factor (vWF) to a small peptide ligand, Ac-RVRSFYK, immobilized on Toyopearl resin, shows that the association constant increases from 8.82×10^5 to 2.06×10^6 M^{-1}, and the maximum capacity from 2.32 to 10.33 mg/ml when the peptide density increases from 32 to 60 mg/ml (Huang et al., 1996).

Baumbach and Hammond (1992) first demonstrated the availability to use peptide ligands as affinity ligands in large-scale chromatography processes by using streptavidin as target. Since then, this technique has been successfully used to purify a variety of proteins, such as s-protein (Huang and Carbonell, 1995), von willebrand factor (Huang et al., 1996), fibrinogen (Buettner et al., 1997; Mondorf et al., 1998), factor IX (Buettner et al., 1996), factor VIII (Necina et al., 1998; Amatschek et al., 2000; Chen et al., 2000), trypsin (Makriyannis and Clonis, 1997), IgG (Verdoliva et al., 1995; Fassina et al., 1996), IgA (Palombo et al., 1998), IgE (Palombo et al., 1998), IgM (Palombo et al., 1998), anti-

MUC1 antibodies (Murray et al., 1997; Murray et al., 1998), Alpha-1-proteinase inhibitor (Bastek et al., 2000) and α-lactalbumin (Gurgel et al., 2001).

Materials and Methods

Peptide synthesis

Two hexamer one-bead-one-peptide libraries were used in our test. Both libraries were synthesized similarly. The first library was from Peptides International (Louisville, KY) and was synthesized onto Toyopearl AF Amino 650M resin of 65μm in diameter with 16 of 20 natural amino acids (except cysteine, methionine, isoleucine and Tryptophan). The second hexamer peptide library was from Bayer Corporation (Raleigh, NC) and was synthesized directly onto Toyopearl AF Amino 650M resin of 120μm in diameter (TosoHass, Montgomeryville, PA) with 18 of the 20 natural amino acids (excepting cysteine and methionine). Both libraries were synthesized using standard fluorenylmethyloxycarbonyl (Fmoc) chemistry as described by Buettner et al. (Buettner et al., 1996, 1998). The base resin is highly hydrophilic with pores of 1000A in diameter, making it suitable for purifying proteins up to 5M Da in size. Briefly, a 1:1 mixture of Fmoc-L-alanine to tBoc(tert.butyloxycarbonyl)-L-alanine was coupled to the amino functionality on the resin. The tBoc group was released with TFA and the free amino functionality was acetylated with acetic anhydride. No further peptide synthesis occurred at these acetylated sites. Subsequently, the Fmoc protecting groups were released with piperidine. Fmoc-protected amino acids were used to synthesize peptides onto resin by using "split synthesis" until the last cycle was finished. The final substitution density based on total amino acid analysis was 100μmol/g. Sequencing of the peptides was performed at the Protein Technology Laboratory, Texas A&M University, College Park, TX, by Edman degradation using a Hewlett Packard G1005A. Identified peptide sequences from primary screening were synthesized directly onto Toyopearl AF 650M

resin at a substitution density of 100μmol/g from Peptides International (Louisville, KY). All the remaining amino groups used to couple peptides on the base resin were acetylated.

Radiolabeling of SEB

Highly purified SEB of at least 95% purity (Toxin Technology, Sarasota, FL) was labeled at 4°C with ^{14}C by reductive methylation utilizing sodium cyanoborohydride and ^{14}C-formaldehyde (NEN Life Science Products, Boston, MA) as described by Jentoft and Dearborn (1983). The sodium cyanoborohydride was recrystallized prior to use due to hydrate formation during storage. SEB was dissolved into 0.1M sodium phosphate buffer, pH 7.0. Sodium cyanoborohydride and ^{14}C-formaldehyde were added sequentially. The ^{14}C-formaldehyde was added at a 2-fold molar excess over 5% of the total methylation sites (68 total, 2×(33 lysines + N-terminus)) to achieve around 5% methylation of SEB. Sodium cyanoborohydride was added at 10X the molarity of ^{14}C-formaldehyde. The reaction was performed at 4°C overnight. The labeled SEB was separated from ^{14}C-formaldehyde by using an EconoPac 10DG Desalting Column (Biorad, Hercules, CA) equilibrated with 0.1M sodium phosphate buffer, pH 7.0. The radioactivity of each fraction collected during the separation was determined using a 1500 Tri-Carb Liquid Scitillation Analyzer (Packard, Meridian, CT) and CytoScint ES scintillation liquid (ICN, Mesa, CA). The protein concentration of each fraction was quantified by the BCA Assay (Pierce, Rockford, IL). The radioactivity yield after labeling was around 10^{14}~10^{15} dpm/mole SEB.

Primary screening of the libraries

A radiological detection approach described by Mondorf et al. was used to deduce the peptides that bind SEB (Mondorf et al., 1998). 20mg of peptide library beads were washed with 20% methanol for one hour, and then washed thoroughly with the binding buffer, phosphate buffered saline (PBS) (Sigma, St. Louis, MO) containing 10mM phosphate buffer, 2.7mM KCl, and 137mM NaCl, pH 7.4. The blocking reagent used for the beads from the first library was a mixture of 1% casein (Pierce, Rockford, IL), 1% BSA (Pierce, Rockford, IL) and *E. coli* lysate (1X over SEN in mass) (Promega, Medison, WI), while the blocking reagent used for the beads from the second library was 1% casein or 0.5% casein plus 0.5% BSA. The library beads were first blocked with 1ml of blocking reagent in PBS in a 1.7ml centrifuge tube for 2 hours on a rotating plate to minimize nonspecific interactions between the library beads and ^{14}C-labeled SEB. Concentrated ^{14}C-labeled SEB was added directly into the reaction slurries to reach a concentration of 0.5μM and incubated for 2 hours at room temperature. Following the incubation, the beads were transferred to a Poly-Prep chromatography column (Biorad, Hercules, CA) and washed thoroughly with PBS with 0.05% Tween (Sigma, St. Louis, MO) until the radioactivity of the flow through reached background levels. The washed beads were suspended into 0.8% low melting agarose (Biorad, Hercules, CA) at 35°C and divided equally into two aliquots in 50ml containers. Each of slurry was poured onto a 160×180 mm gelbond (BioWhittaker Molecular Applications, Rockland, ME) to form a monolayer of beads. The gelbond was air-dried overnight in a hood and subsequently exposed to Kodak Biomax MR autoradiography film (Fisher, Atlanta, GA) for 5 days. Positive signals on the film were confirmed by reexposure of the gelbond to a new film.

Beads corresponding to the positive signals were identified by careful alignment of the films and gel, and isolated with a scalpel under a microscope. The isolated beads were each placed in a centrifuge tube with water and incubated for 10 minutes at 75°C in a water bath, followed by sonication for 10 minutes to remove the agarose from bead. The clean beads were suspended in methanol and sent to Protein Technologies Laboratories, Texas A&M University, TX, for sequencing by Edman degradation using a Hewlett-Packard G1005A.

Secondary screening: binding confirmation in batch format

Non-competitive format

Individual peptides deduced from primary screening were synthesized directly onto Toyopearl AF Amino 650M resin at a substitution of 100μmol/g resin (Peptides International, Louisville, KY). All remaining amino groups were acetylated. Beads were washed with 20% methanol for one hour and subsequently rinsed thoroughly with PBS buffer. 400μl of the slurry in PBS with a bead density of 1mg/20μl was transferred to a 0.5ml centrifugal filter with 0.45μm Durapore membrane (Millipore, Milford, MA). Buffer was removed by centrifugation. Resins were incubated with 400μl of 1μM ^{14}C-SEB in PBS buffer at room temperature on a rotating plate for 2 hours, and then washed with PBS buffer for 15 min, and then sequentially washed for one hour each with 400μl of: 1M NaCl in PBS buffer, 2% acetic acid and 6M GdnHCl. Each solution after centrifuge was collected into scintillation fluid for radioactivity counting. The total unbound ^{14}C-SEB is determined by combining the counts of unbound ^{14}C-SEB and the wash of PBS. Finally, resins were suspended in PBS buffer and counted for radioactivity

in the same manner, allowing a total radioactivity balance. Toyopearl AF Amino 650M resins and acetylated Toyopearl AF Amino 650M resins were treated the same as above as controls.

Competitive format

Positive peptides identified from the non-competitive format studies were incubated with a mixture of labeled SEB and a blocker protein to test the affinity of the peptides to SEB. The procedure was similar to that of non-competitive format except using a mixture of SEB and blocker instead of SEB, or adding blocker first to block the peptides for one hour and then replacing blocker with the mixture. Two blockers including 1mg/ml BSA (Pierce, Rockford, IL) and 1mg/ml casein (Pierce, Rockford, IL) were tested for their effects on SEB binding. Toyopearl AF Amino 650M resins and acetylated Toyopearl AF Amino 650M resins were investigated the same as above as controls.

Tertiary screening: binding confirmation in column format

To test the selectivity of the peptides that showed positive binding in the secondary screening, the performance of the peptide ligands was investigated in a chromatography column format. In order to minimize the usage of SEB due to its high costs and toxicity, the peptide beads were packed into a 0.21×3cm column, a microbore column from Alltech (Deerfield, IL) and tested on the Walters 616 LC system (Millipore, Milford, MA) with a UV detector (Knauer, Germany) at 280 nm. The column was pre-equilibrated with binding buffer at a flow rate of 0.15ml/min (173cm/hr). The following program was used for each column run: From time 0 to 10 minutes, sample was injected through a 100µl loop (Thomson, Springfield, VA) at a flow rate of 20µl/min. The column

was then washed with binding buffer for 10 minutes and subsequently with 1M NaCl in binding buffer for 20 minutes at a flow rate of 0.15ml/min. From time 40 to 60 minutes, the bound protein was eluted by switching to 2% acetic acid at a flow rate of 0.15ml/min. The column was re-equilibrated with 50 CVs (column volume) of binding buffer before the next run. *E. coli* lysate (Promega, Medison, WI) and BSA(Pierce, Rockford, IL) were used as competitive protein mixtures to test the selectivity of the ligands to SEB in chromatography. Using 0.5M sodium chloride in PBS as binding buffer, several solutions of *E. coli* lysate combined with SEB were tested: 1mg/ml *E. coli* lysate, 1mg/ml *E. coli* lysate + 0.25mg/ml SEB, 1mg/ml *E. coli* lysate + 0.5mg/ml SEB, 1mg/ml *E. coli* lysate + 1mg/ml SEB, 1mg/ml SEB. Similar tests were done except using 5mg/ml BSA instead of 1mg/ml *E. coli* lysate. All peaks were collected and their composition determined by Sodium-dodecyl-sulfate polyacrylamide gel electrophoresis (SDS-PAGE) (Invitrogen, Carlsbad, CA). We also investigated the recovery of 0.5mg/ml of SEB from various amount of *E. coli* lysate (1mg/ml, 2.5mg/ml, 5mg/ml, and 10mg/ml).

To investigate the effect of salt on the separation of SEB from *E. coli* lysate, another chromatography run was used. From time 0 to 10 minutes, sample was injected through a 100µl loop (Thomson, Springfield, VA) at a flow rate of 20µl/min. The column was then washed with binding buffer for 10 minutes and subsequently with 1M NaCl in binding buffer for 35 minutes at a flow rate of 0.15ml/min. From time 55 to 75 minutes, the bound protein was eluted by switching to 2% acetic acid at a flow rate of 0.15ml/min. The column was re-equilibrated with 50 CVs (column volume) of binding buffer before the next run.

Results

Primary screening

Initial work in the identification of peptide ligand for SEB was performed by using library 1, a one-bead-one-peptide library from Peptides International. This peptide library was made of 16 of 20 natural amino acids (except cysteine, methionine, isoleucine and Tryptophan) and the diameter of the library beads was 65μm on average. The blocking agent used was a mixture of 1% casein and 1% BSA and *E. coli* lysate (10X over SEB in mass). 0.5μM of labeled SEB was used to yield enough background for alignment after 5 days of exposure of the beads to photographic films. There were no positive beads whose signals were well above the background. Thirty-five beads that had small positive signals were picked out and sequenced. The sequences are listed from the N to C terminus as shown in Table 2. Because high density of peptide ligands would cause significant high background due to nonspecific interaction, the peptide library used here was a low-density library at a density of 100μmol/g resin. The amount of amino acid at each cycle is less than 10pmol/bead, while the limitation of detection is 5~10pmol/bead by using Edman degradation at Texas A&M. This is a part of reason resulting in difficulty in sequencing. In addition, the beads of 65μm are so small that it's hard to handle them while picking them out from gels and washing them to remove the agarose. Thus another one-bead-one-peptide library (library 2) containing larger beads was used next to determine peptide ligands for SEB.

The second library was synthesized as described by Buettner et al. using 18 of 20 amino acids (excepting cysteine and methionine) at the same density as that of the first one but with larger beads (120μm). It was expected that larger beads would have more

peptides in each bead and hence yield stronger signals than small beads. Compared to the first library, the second library produced positive signals well above the background, and it was much easier to handle these larger beads. Around 1gram of library beads were screened, which represents 5% of a whole peptide library. Unfortunately, many positive beads selected for sequencing have truncated sequences due to the low peptide density. Usually the first three amino acids near the N terminus can be sequenced, and tyrosine (Y) and tryptophan (W) dominate these positions (Table 5). The initial yield of the first amino acid on the bead ranges from 3.8 to 21.1pmol indicating that there is a large distribution of peptide density on beads of this library. This peptide library had been used previously to deduce peptide ligands for ovalbumin and prion protein in our lab. Thus the problems associated with low peptide density and large distribution of peptide density may result from long-time storage (received in Nov., 21, 1996). The peptide sequences with all 6 amino acids sequenced are listed from the N to the C terminus in Table 4. Aromatic amino acids dominate the first three positions at the N terminus in peptides No.7 to No.12; especially there is a consensus, YYW, in sequences No. 7, 8, and 9. In addition, there is another consensus, WHH, near the C terminus in sequences No.9 to No.12, and a similar sequence to WHH, WLH, in sequence No.8. These sequences indicate that aromatic amino acids may play a critical role for SEB binding.

Secondary screening

Secondary screening was used to confirm the peptide ligands from primary screening. 14 of 35 peptide sequences deduced from the first library and 12 of peptide sequences from the second library were synthesized directly onto Toyopearl AF Amino 650M resins through its C-end at a density of 100μmol/g-resin. The remaining free amino

groups at the bead surface were acetylated to avoid nonspecific binding. Because it was difficult to call the first cycle of the peptide P/Y-Y-W-L-H-H, both PYWLHH and YYWLHH were synthesized for secondary screening. All peptide ligands were incubated with 1μM of ^{14}C-labeled SEB for 2 hours to make sure equilibrium was established. The bound SEB was eluted by using 1M NaCl, 2% acetic acid, and 6M GdnHCl. Nonspecific electrostatic interactions were removed by using 1M NaCl. Stronger binding due to affinity would be disrupted by using protein denaturant, 2% acetic acid and 6M GdnHCl. Results are shown in Table 6 and Table 7 with blank Toyopearl AF Amino 650M resin and its acetylated form as controls. None of the sequences that deduced from the first library can bind SEB significantly. This might have been expected because it was much more difficult to pick up positive signals from background signals in primary screening. There are only two peptide sequences, YYWFYY and YYWLHH (YYWHHG will be tested in future), which can bind most of the SEB added, although other peptide sequences such as FYYLPE, PYWLHH, YWHHHD and YIWHHI have similar structure to these two. This indicates that the first three amino acids, YYW, may be special for SEB recognition. Goldman E.R. et al. have deduced several peptide sequences that bind SEB from a 12-mer phage-display library and there is a consensus, WHK, at the N terminus of these sequences (Goldman E.R. et al., 2000)(Table 12). We selected one of them, WHKAPRAPAPLL, and synthesized its first 6 amino acids near the N terminus, WHKAPR, onto Toyopearl AF Amino 650M resin to test its ability to bind SEB. As shown in Table 7, it is no better than the control resins. In order to choose an appropriate concentration of SEB used in secondary screening, some of secondary screening experiments were performed using 0.5μM or less SEB, and the percentage of the bound

SEB was increased on both control resins and tested peptide ligands (data not shown). In this case, a significant portion of the binding was due to the nonspecific interaction between SEB and the solid support. Thus 1μM of SEB was set up for further investigations so that the nonspecific binding onto the support can be neglected.

In order to know the selectivity of the positive peptide ligands, YYWFYY and YYWLHH, competitive secondary screening was performed by adding competitive proteins with SEB into binding buffer. Two kind of proteins, casein and BSA, which were the blockers used in the primary screening, were tested. The results are presented in Table 8 with Toyopearl AF Amino 650M resin and its acetylated form as controls. The binding capacities of both YYWFYY and YYWLHH decreased with the existence of a competitive protein. Casein was a stronger blocker than BSA to inhibit SEB binding. The performance of YYWLHH was much better than that of YYWFYY. Even though the concentration of competitive protein was 35 times higher than that of SEB, YYWLHH was still able to bind more than 60% of SEB. This indicates that the amino acids close to the resin also affected the performance of the peptide ligand significantly although steric effects inhibited their contact with binding molecules. We further tested the affinity of peptide ligands to SEB by blocking the peptides first using casein or BSA, then added SEB directly to the SEB solution to see the performance of SEB binding. The fractions of bound SEB were almost the same for YYWFYY and YYWLHH, but dropped dramatically in comparison with those in noncompetitive secondary screening (Table 9). This shows that casein and BSA also blocked the specific binding sites for SEB besides blocking nonspecific binding sites, and SEB was not strong enough to displace bound casein or BSA during incubation time in our experiments (2 hours).

The addition of a nonionic detergent, Tween 20, into binding buffers made the peptides YYWFYY and YYWLHH lose their ability to bind SEB (Table 10). The effect of salt was also investigated in batch format (Table 11). Two peptide ligands with similar structure to positive ligands, YIWHHI and FYYLPE, and Toyopearl AF Amino 650M resin and its acetylated form were used as controls. The addition of 1M NaCl into binding buffer had positive effects on the binding of SEB onto YYWFYY, YYWLHH and YIWHHI, but had little effect on the binding of SEB onto FYYLPE, Toyoperal Amino resin and its acetylated form. Thus the effect of salt was not universal even though the peptide ligands had similar hydrophobicity.

Tertiary screening

We chose peptide ligand YYWLHH as the specific ligand for SEB in chromatography tests, and amino resins, acetylated resins, FYYLPE and YIWHHI as controls. The results of SEB binding in chromatography were similar to those in batch system. SEB was eluted only by 2% acetic acid in the column of YYWLHH, while most of SEB injected in control columns remained in flow through and 1M NaCl fractions before the application of 2% acetic acid (Figure 3). We also investigated SEB binding using different amounts of salt (NaCl) in binding buffer in order to know if nonspecific hydrophobic interactions could contribute to SEB binding. As shown in Figures 4 and 5, the fraction eluted by 1M NaCl in PBS buffer disappeared when increasing NaCl to 0.5M in binding buffer for amino resins, while other columns seemed to be little affected by salt.

In order to further demonstrate the affinity of YYWLHH to SEB, a protein mixture, *E. coli* lysate, and a blocking reagent used in primary screening, BSA, were used as the competitive proteins for SEB in chromatography. Although salt had little effect on SEB binding (Figure 6), the retention of *E. coli* lysate in YYWLHH was sensitive to salt concentration in binding buffer (Figure 7) and therefore affected the separation of SEB from *E. coli* lysate (Figure 8). The materials represented by the peak at 40min in Figure 8 eluted by 1M NaCl in PBS seemed to prevent/weaken SEB binding when PBS was used as binding buffer. This peak disappeared and SEB binding was restored when the salt concentration was increased to 0.25M or more in binding buffer. Similar studies on the binding of *E. coli* lysate to two control columns, amino and acetylated amino columns, showed that there was no such a peak, indicating that the appearance of this peak was due to the attachment of peptides YYWLHH to the resins (Figure 9 and 10). All materials in *E. coli* lysate flew through the acetylated amino column (Figure 10). Some components in *E. coli* lysate bound to the amino column and were released by using 1M salts when the binding buffer contained small amount of salts. The increase of the salt concentration in the binding buffer decreased the binding capacity of the amino column indicating that nonspecific ionic interactions contributed to the binding of *E. coli* lysate. The amino column lost its ability to bind *E. coli* lysate totally when the salt concentration increased to 0.5M (Figure 9). Thus 0.5M NaCl in PBS buffer was used as binding buffer later on to eliminate nonspecific ionic interactions while not causing much hydrophobic interaction.

The purification of different amounts of SEB from 1mg/ml of *E. coli* lysate on the column YYWLHH is presented in Figure 11. Most of the components in *E. coli* lysate were removed by the wash with binding buffer and 1M NaCl, and SEB was only eluted

with 2% acetic acid. The area under the 2% acetic acid peak increased by the same proportion as the increase in SEB concentration in the injected mixture, indicating that all of the injected SEB was released by 2% acetic acid. The recovery of SEB from *E. coli* lysate was almost same as that in noncompetitive chromatography runs (Figure 11). There was a small and tailing peak after the toxin peak and further electrophoresis showed that it represented some components in *E. coli* lysate adsorbed to the column more strongly. SDS-PAGE analysis was done on the fractions collected during the chromatography separation (Figure 12). SEB was concentrated in the 2% acetic acid fraction. The remaining fractions collected unbound proteins (flow through peak), proteins bound with electrostatic interactions (1M NaCl peak), and tightly bound proteins (the peak after SEB peak). The band corresponding to proteins in 1M NaCl fraction was undetectable even after 10 times concentration, indicating that the protein concentration might be very low or the peak of 1M NaCl fraction might result from DNA or RNA in *E. coli* lysate. Another run of all the fractions in gels for nucleic acids did find several clear bands in both flow through and 1M salt fractions (data not shown). In order to know further about the ability of column YYWLHH to recover SEB, we increased the concentration of *E. coli* lysate in the mixture with 0.5mg/ml SEB. Compared to the noncompetitive chromatography, the addition of *E. coli* lysate up to 2.5mg/ml had little effect on the toxin binding. The area of the toxin peak dropped to 70% and 50% when the concentration of *E. coli* lysate increased to 5mg/ml and 10mg/ml, respectively (Figure 13), and some of the SEB came out in flowthrough and 1M salt fractions demonstrated in electrophoresis (Figure 14).

A series of chromatography runs was done similar to the methods used to generate Figure 11 except using 5mg/ml of BSA instead of 1mg/ml of *E. coli* lysate as competitive protein. As in Figure 15, most of BSA flows through the column with the binding buffer. There was nothing coming out in 1*M* sodium chloride. Some of BSA retained on the column strongly and came out after SEB in 2% acetic acid. The area under the first 2% acetic acid peak increased by the same proportion as the increase in SEB concentration in the injected mixture, indicating that SEB was concentrated in this peak. Electrophoresis results showed that the component in the first 2% acetic acid peak was as same as SEB standard and no BSA contaminated this fraction (Figure 16). All injected BSA could not bind to control columns, amino and acetylated amino columns (Figure 17). Therefore, the bound BSA released in 2% acetic acid was due to the attached peptide.

Discussion

A low-density peptide library at a density of 100 μmol/g resin was used in this study to minimize nonspecific interactions. Although both casein and BSA were used as blocking reagent in our work, casein is a better blocking reagent than BSA since BSA also lowered the number of positive signals significantly.

The first library we used had small beads (65μm in diameter), which resulted in difficulties in handling these beads. Small beads also yielded a low ratio of signal-to-noise that caused difficulty in picking up positive signals. Furthermore, the amount of peptides on each bead was close to the detection limit of Edman degradation. This prevented identification of the exact amino acid at each cycle. The library that had larger particles of resin (120μm) facilitated bead selection and sequencing. This library had a large range of distribution of peptide density (from 3.8 to 21.1pmol on each bead). Some signals coming from nonspecific interactions due to relatively higher peptide density on the bead were well above the background and treated as positive signals. In addition, this library seemed to have a large number of truncated sequences.

No consensus sequence was apparent from the peptide sequences deduced from the first library. Hydrophobic amino acids dominated the cycles close to the N terminus (Table 3). None of the peptide ligands selected for secondary screening from the first library bound SEB significantly probably due to the problems associated this library. Most of the peptide sequences (including the truncated sequences) deduced from the second library had aromatic amino acids near the N terminus, but sequences YYWFYY and YYWLHH exhibited stronger binding, indicating YYW at the N terminus may be specific for SEB recognition. Further competitive secondary screening demonstrated that

YYWLHH was more specific for SEB than YYWFYY suggesting that the amino acids close to the C terminus also seem to contribute to the affinity of the ligand. The consensus sequence WHK in peptide sequences deducted from a phage library by Goldman E.R. et al. (2000) (Table 12) is similar to the consensus sequence WHH in some peptides deduced from the second library, but neither WHK nor WHH were able to bind SEB without YYW. Sato et al. also have demonstrated that aromatic amino acids in peptide ligands are essential to bind TSST-1, a Staphylococcal enterotoxin similar to SEB in 3D structure (Sato et al., 1996). The peptide ligands deduced from a phage library for SEB also have an aromatic amino acid, tryptophan, at the N terminus (Goldman et al., 2000) although the binding capacity of one of them was low in our tests. More importantly, the aromatic consensus we found at the amino end, YYW, from the second library is necessary to capture most of the SEB.

Part of one of the sequences including the consensus (WHK) deduced from a phage library (Table 12) were coupled to Toyopearl resin to test its availability for SEB binding. Unfortunately, the binding results in secondary screening were negative. We don't know the exact reasons for that. Probably other amino acids besides the consensus are essential for SEB binding. On the other hand, maybe the binding of SEB is sensitive to the change of the solid support from phage to Toyopearl resin. Murray et al. found similar results for the purification of anti-MUC1 antibody where the hexamer peptides KSKAGV from a phage library couldn't capture the antibody when the sequence was linked to a Sepharose matrix (Murray et al., 1997).

The study of fibrinogen bound to FLLVPL demonstrated that electrostatic interactions are essential in the initial adsorption process, and then hydrophobic

interactions will strengthen the binding thereafter (Hentsch et al., 1996). SEB is positively charged in PBS binding buffer (pI 8.6 > pH 7.4), and one would think that negatively charged amino acids at the N terminus would facilitate SEB binding. But both ligands, YYWFYY and YYWLHH are positively charged due to the free amino groups at the N terminus. Thus hydrophobic interactions seem to control the binding of SEB. This has been confirmed both in secondary screening (Table 11) and chromatography (Figure 8) by adding salts to favor SEB binding. In addition, another proof for that is that the fraction of bound SEB was reduced significantly while using PBS with Tween (0.05%) as binding buffer because detergent is usually used to disrupt/prevent hydrophobic interactions (Table 10). Moreover, the hydrophobic interactions between SEB and positive peptide ligands are specific because other similar peptide ligands in hydrophobicity didn't bind SEB even with 1M NaCl (Table 11).

The performance of YYWLHH in chromatography experiments verified the specificity to SEB demonstrated in batch system. The acetylated amino column cannot retain SEB any more. Only a small amount of SEB was retained on Toyopearl amino resins nonspecifically and able to be eluted by using 1M NaCl. The interaction between the bound SEB and amino resins should be primarily electrostatic. Although SEB is positively charged in binding buffer, probably the charge distribution is not uniform and there are still some negative-charge concentrated areas at the SEB surface, which can interact with amino groups on the amino resins. Due to the immobilization of YYWLHH on Toyopearl amino resin, the column was able to retain SEB and the bound SEB was eluted only by a protein denaturant, 2% acetic acid. Since aromatic acids, YYW, at the N terminus of YYWLHH determined SEB binding, it is possible that the binding of SEB

was due to nonspecific hydrophobic interactions. Thus another two hydrophobic peptides, FYYLPE and YIWHHI were used as controls. Their performances were similar to amino resin (Figure 3). In addition, increasing salt concentration had little effect on the binding of SEB to FYYLPE and YIWHHI (Figures 4 and 5). Therefore, the interactions between SEB and YYWLHH are specific.

The specific interaction between SEB and YYWLHH was further investigated by the separation of SEB from *E. coli* lysate and BSA. The interactions between SEB and YYWLHH are not as specific as antigen-antibody interactions. The attachment of YYWLHH to chromatography resins also brings some nonspecific interactions. When using PBS as binding buffer, some components in *E. coli* lysate inhibited SEB binding. The binding of such components seemed to be due to electrostatic interactions since they were removed by using 1M NaCl. The effect of such nonspecific binding on SEB binding could be avoided by adding extra salt in binding buffer (Figure 8). In general, ionic interactions will be diminished with no more than 0.4M sodium chloride in solution, while hydrophobic interactions require more than 1M salt in the binding buffer (Huang and Carbonell, 1995). Thus 0.5M NaCl in PBS buffer was used as binding buffer later on to achieve efficient recovery of SEB. Although a small fraction of both *E. coli* lysate and BSA bound the column strongly and had to be eluted by 2% acetic acid, the recovery of SEB was little affected even when the concentration of competitive protein was increased up to ten times that of SEB (Figures 13 and 15). In addition, SEB came out earlier than strongly bound either *E. coli* lysate or BSA in elution buffer (2% acetic acid) indicating that they could have different binding sites on the column. The electrophoresis results

show a uniform composition of the toxin peak (Figures 12, 14, and 16), so that a successful separation of SEB from either *E. coli* lysate or BSA has been achieved.

Conclusions

An affinity peptide ligand for SEB, YYWLHH, has been derived from a solid phase combinatorial peptide library. We have demonstrated a successful purification of SEB from a mixture of SEB with *E. coli* lysate or BSA in one step using this peptide ligand. In addition, this short peptide could help us understand the interactions of SEB with its natural ligands, such as TCR and MHCII.

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Table 1: Methods used in the purification of SEB described in the literature.

Table 2: Sequences deduced from the first library. In each run of screening, 20 mg of the first hexametric, solid phase peptide library was blocked with 1% casein combined with 1% BSA and *E. coli* lysate (10X over SEB in mass) first for 2 hours in PBS buffer, pH 7.4. Then ^{14}C labeled SEB was added directly into reaction solution to reach a concentration of 0.5μM and incubated for another 2 hours. Following the screening, the beads were suspended into 0.8% agarose and then poured on gelbond to form a monolayer and exposed to autoradiography film for 5 days. 35 beads were isolated and sequenced by Edman degradation. Sequences are listed from the N to C terminal positions.

Table 3: Sequence analysis of peptides from the first library. Most frequently occurred amino acids at each cycle are listed by position from the N to C terminus based on the sequences listed in Table 2.

Table 4: Sequences deduced from the second library. In each run of screening, 20 mg of the first hexametric, solid phase peptide library was blocked with 1% casein or 0.5% casein plus 0.5% BSA first for 2 hours in PBS buffer, pH 7.4. Then ^{14}C labeled SEB was added directly into reaction solution to reach a concentration of 1 μM and incubated for another 2 hours. Following the screening, the beads were suspended into 0.8% agarose and then poured on gelbond to form a monolayer and exposed to autoradiography film for 5 days. Sequences are listed from the N to C terminal positions.

Table 5: Truncated sequences deduced from the second library. The conditions and methods of the primary screening were the same as those in Table 4. Cycles where the amino acids cannot be determined are denoted with "X".

Table 6: Secondary screening of peptides from the first library. 20 mg of peptide beads were incubated with 1 μM of ^{14}C labeled SEB in PBS buffer, pH 7.4, for 2 hours. Following incubation, beads were washed with PBS for 15 minutes, and then sequentially washed for 1 hour with: 1M NaCl in PBS, 2% acetic acid, and 6M GdnHCl. The total unbound ^{14}C-SEB was determined by combining the counts of unbound ^{14}C-SEB and the wash of PBS. Finally, resins were suspended in PBS buffer and counted for radioactivity in the same manner, allowing a total radioactivity balance.

Table 7: Secondary screening of peptides from the second library. 20 mg of peptide beads were incubated with 1 μM of ^{14}C labeled SEB in PBS buffer, pH 7.4, for 2 hours. Following incubation, beads were washed with PBS for 15 minutes, and then sequentially washed for 1 hour with: 1M NaCl in PBS, 2% acetic acid, and 6M GdnHCl. The total unbound ^{14}C-SEB was determined by combining the counts of unbound ^{14}C-SEB and the wash of PBS. Finally, resins were suspended in PBS buffer and counted for radioactivity in the same manner, allowing a total radioactivity balance.

Table 8: Competitive secondary screening. 1 μM of ^{14}C labeled SEB with competitive proteins, 1mg/ml of casein or BSA, was incubated with 20 mg beads in PBS buffer, pH 7.4, for 2 hours. Following incubation, beads were washed with PBS for 15 minutes, and

then sequentially washed for 1 hour with: 1M NaCl in PBS, 2% acetic acid, and 6M GdnHCl. The total unbound ^{14}C-SEB was determined by combining the counts of unbound ^{14}C-SEB and the wash of PBS. Finally, resins were suspended in PBS buffer and counted for radioactivity in the same manner, allowing a total radioactivity balance. Non-competitive format of secondary screening was used as control.

Table 9: Secondary screening with blocked peptide ligands. 20 mg of beads were blocked with 1 mg/ml casein or BSA for 2 hours. Then ^{14}C-SEB was added directly into the reaction solution to reach a concentration of 1 μM. Following 2 hours of incubation, beads were washed with PBS for 15 minutes, and then sequentially washed for 1 hour with: 1M NaCl in PBS, 2% acetic acid, and 6M GdnHCl. The total unbound ^{14}C-SEB was determined by combining the counts of unbound ^{14}C-SEB and the wash of PBS. Finally, resins were suspended in PBS buffer and counted for radioactivity in the same manner, allowing a total radioactivity balance. Non-competitive format of secondary screening was used as control.

Table 10: Effect of Tween on the binding of SEB to YYWFYY and YYWLHH. 20 mg of peptide beads were incubated with 1 μM of ^{14}C labeled SEB in PBS buffer with 0.05% Tween 20, pH 7.4, for 2 hours. Following incubation, beads were washed with PBS with 0.05% Tween 20 for 15 minutes, and then sequentially washed for 1 hour with: 1M NaCl in PBS with 0.05% Tween 20, 2% acetic acid, and 6M GdnHCl. The total unbound ^{14}C-SEB was determined by combining the counts of unbound ^{14}C-SEB and the wash of PBS with 0.05% Tween 20. Finally, resins were suspended in PBS buffer with 0.05% Tween

20 and counted for radioactivity in the same manner, allowing a total radioactivity balance. The same runs using PBS as binding buffer were used as controls.

Table 11: Effect of salt in secondary screening. 40 mg of beads were divided into two aliquots. One was incubated with 1 μM of ^{14}C-SEB in PBS buffer, pH 7.4, the other in PBS buffer with 1M NaCl, pH 7.4, both for 2 hours. Beads were washed with corresponding binding buffer for 15 minutes. The total unbound ^{14}C-SEB was determined by combining the counts of unbound ^{14}C-SEB and the wash of PBS. Resins were suspended in PBS buffer and counted for radioactivity in the same manner, allowing a total radioactivity balance.

Table 12: SEB binding sequences isolated from a phage display library (Goldman et al., 2000).

Table 1: Methods used in the purification of SEB

Source	Procedure	Recovery (%)	Purity (%)	Homogeneity
Bergdoll, et al., 1959, 1961	Acid precipitation; Adsorption on Amberlite IRC-50; Ethanol precipitation; Starch-bed electrophoresis	~0.04 Only mg quantities obtained	Highly purified	Nearly homogeneous
Frea et al., 1963	Ethanol precipitation; Gel filtration using Sephadex G-100; Electrophoresis on Sephadex	~0.08% Only mg quantities obtained	Partial purified	Nearly homogeneous
Schantz et al., 1965	2× Cation-exchange using CG-50 resin; Cation-exchange using CM-cellulose;	50-60	~99	Homogeneous in size; Microheterogeneity in charge
Ende et al., 1983	Cation-exchange using CG-50 resin; Chromatofocusing; Sephadex G-50	60	100; No contaminants detected	Homogeneous in size; Microheterogeneity in charge
Melconian et al., 1983	Cation-exchange using Biorex 70 resin; Isoelectric focusing; Sephadex G-100	9	99	Homogeneous in size and charge
Williams, et al., 1983	Size-exclusion chromatography using MicroPak TSK 3000SW		Poor resolution of SEB	
	Cation-exchange chromatography using MicroPak TSK IEX 530CM		Contaminated with low-molecular-weight species	
	Reversed phase chromatography using MicroPak Protein-C_{18}		Contaminated with low-molecular-weight species	

Table 1 (continued)

Strickler et al., 1989	Reverse-phase chromatography using DeltaPak C_{18}; Cation-exchange chromatography using Protein Pak SP-5PW	45	Contaminated with low-molecule-weight toxin material	Homogeneous in Gel filtration HPLC analysis
Brehm et al., 1990	Cross-flow filtration; Dye ligand Chromatography using Red A	44	99	Homogeneous in size; Microheterogeneity in charge
Johansson et al., 1990	Cation exchange using S Sepharose; Cation exchange using S Sepharose conducted on BioPilot; Gel filtration using Superdex 75 conducted on BioPilot	74	~99	Homogeneous in size;
Bhatti et al., 1994	$(NH_4)_2SO_4$ precipitation; Hydrophobic chromatography using Phenyl-Sepharose; Chromatofocusing; SephadexG-25	56	100; No contaminates detected	Homogeneous in size; Microheterogeneity in charge
Lopes et al., 1996	Cation-exchange using CG-50 resin; Dye ligand Chromatography using Red A	59	~99	Homogeneous in size

Table 2: Sequences deduced from the first library

	Sequence					
1	G	L	Q	E	F	T
2	A	S	Y	L	G	G
3	P	N	F	F	G	L
4	I	S	G	V	F	P
5	F	D	V	G	K	D
6	X	F	T	S	N	Q
7	E	V	V	Y	K	L
8	W	Y	Q	K	F	G
9	X	Y	V	L	K	V
10	V	L	Y	K	V	G
11	No sequences detected					
12	No sequences detected					
13	P	K	G	K	V	A
14	A	V	Y	Y	F	K
15	A	N	P	K	W	G
16	E	E/H	S	K	F	L
17	Q	D	F	G	G	V
18	G	G	P	L	G	F
19	E	P	E	G	T	D
20	Y	G	Y	T	G	S
21	A	T	V	N	G	D
22	H	Y	D	P	G	A
23	A	Y	A	H	Y	A
24	H	S	A	V	G	D
25	No sequences detected					
26	A	T/H	L	G	F	R
27	A	G	Y	F	G	Q
28	X	F	Q	P	V	T
29	P	F	L	H	G	D
30	H	D	H	A	V	L
31	A	F	S	N	Y	P
32	H	F	V	A	F	V
33	A	Y	F	K	V	P
34	A	N	Y	H	N	N
35	A	F	F	T	Y	F

Table 3: Sequence analysis of peptides from the first library

1	2	3	4	5	6
A	F	V	G	G	G
	Y	F	K	F	L
		Y			D

Table 4: Sequences deduced from the second library

	Sequence					
1	K	L	Q	A	T	I
2	I	Q	I	R	F	G
3	A	Y	F	K	V	P
4	A	F	G	W	W	H
5	V	P	T	Y	S	E
6	F	Y	Y	L	P	E
7	Y	Y	W	F	Y	Y
8	P/Y	Y	W	L	H	H
9	Y	Y	W	H	H	?
10	Y	W	H	H	H	D
11	Y	I	W	H	H	I
12	R	W	W	H	H	H

Table 5: Truncated sequences deduced from the second library

	Sequence					
1	P/F	Y	X	X	X	X
2	P	W	X	X	X	X
3	P	Y	H	H	X	X
4	L	V	V	W	E/P	X
5	W	W	W	X	X	X
6	P	W	V	F	G	X
7	P	W	W	L	P	E/Q/R
8	D/A/P	W/F	W/S	N/L	H/Y	H/P
9	P	Y	W	X	X	X
10	X	F	T	V	T	N
11	P	W	X	E	X	X
12	Y	Y	X	X	X	X
13	P	W	H	Y	X	X
14	V	W	T	H	L	X

Table 6: Secondary screening of peptides from the first library

Peptide	Flow through (% of total)	1M NaCl (% of total)	2% acetic acid (% of total)	6M GdnHCl (% of total)	Beads (% of total)
Control resins					
Amino*	84.54	0.30	0.21	3.20	8.21
Acetylated**	89.85	0.24	0.16	0.12	4.90
Peptide sequences from the first library					
VLYKVG	78.07	0.87	0.80	6.30	14.96
ATVNGD	84.39	1.00	0.46	2.56	11.58
HFVAFV	76.89	0.96	2.37	7.65	12.13
AYFKVP	75.71	0.62	0.89	6.65	16.13
HYDPDA	78.13	0.85	1.12	6.03	13.86
PFLHGD	89.66	0.94	1.45	0.97	6.98
AGYFGQ	83.38	0.89	0.61	2.83	12.29
QDFGGV	85.14	0.76	0.38	1.79	11.92
PKGKVA	84.42	0.39	0.38	9.46	5.35
AHLGFR	91.95	1.85	1.39	3.32	1.49
WYQKFG	89.16	1.59	1.43	4.15	3.67
AFSNYP	85.12	1.27	0.87	8.81	3.92
EVVYKL	87.03	0.96	0.78	6.31	4.91
ASYLGG	85.37	0.68	0.72	8.93	4.31

Table 7: Secondary screening of peptides from the second library

Peptide	Flow through (% of total)	1M NaCl (% of total)	2% acetic acid (% of total)	6M GdnHCl (% of total)	Beads (% of total)
Control resins					
Amino*	84.54	0.30	0.21	3.20	8.21
Acetylated**	89.85	0.24	0.16	0.12	4.90
Peptide sequences from the second library					
KLQATI	82.49	1.43	0.75	12.51	2.82
IQIRFG	85.93	1.25	0.52	9.77	2.53
AYFKVP	75.71	0.62	0.89	6.65	16.13
AFGWWH	94.43	0.54	1.17	1.94	1.92
VPTYSE	96.58	1.68	0.90	0.31	0.52
FYYLPE	95.26	0.60	0.79	1.66	1.70
YYWFYY	**6.39**	**0.65**	**59.93**	**26.14**	**6.89**
YYWLHH	**6.57**	**0.61**	**74.12**	**15.24**	**3.45**
PYWLHH	74.45	0.55	7.06	14.94	3.00
YWHHHD	76.10	5.01	1.62	8.09	9.18
YIWHHI	87.91	1.34	1.36	4.36	5.03
Peptide sequence from phage library					
WHKAPR	88.36	2.83	1.26	4.85	2.70

Table 8: Competitive secondary screening

Peptides	Blocker	Flow through (% of total)	1M NaCl (% of total)	2% acetic acid (% of total)	6M GdnHCl (% of total)	Beads (% of total)
Amino	No blocker	92.63	0.37	1.89	3.37	1.74
	Casein	93.54	3.26	2.13	0.61	0.46
	BSA	95.31	1.40	2.07	0.74	0.48
Acetylated	No blocker	95.23	0.17	0.06	0.33	4.20
	Casein	98.59	1.07	0.07	0.16	0.11
	BSA	98.57	0.86	0.06	0.26	0.25
YYWFYY	No blocker	5.00	0.85	57.95	30.00	6.20
	Casein	47.09	4.49	39.63	6.22	2.57
	BSA	35.35	3.11	44.95	13.03	3.56
YYWLHH	No blocker	4.88	0.51	74.72	15.70	4.19
	Casein	32.06	2.68	56.47	6.36	2.43
	BSA	25.28	2.45	59.03	10.17	3.07

Table 9: Secondary screening with blocked peptide ligands

Resin	Blocker	Flow through (% of total)	1M NaCl (% of total)	2% acetic acid (% of total)	6M GdnHCl (% of total)	Beads (% of total)
Amino	No blocker	94.92	0.42	1.38	2.35	0.94
	Casein	97.02	1.04	1.35	0.27	0.22
	BSA	96.91	1.07	1.35	0.38	0.29
Acetylated	No blocker	95.23	0.17	0.06	0.33	4.20
	Casein	98.00	1.29	0.13	0.27	0.31
	BSA	98.51	0.96	0.06	0.26	0.21
YYWFYY	No blocker	6.42	0.74	59.99	27.49	5.36
	Casein	78.24	4.96	13.59	2.14	1.07
	BSA	53.96	3.54	30.29	10.04	2.16
YYWLHH	No blocker	8.08	0.69	75.58	12.55	3.10
	Casein	78.76	4.42	14.05	1.63	1.14
	BSA	56.79	4.88	32.31	4.36	1.66

Table 10: Effect of Tween on the binding of SEB to YYWFYY and YYWLHH

Resin	W/Wt Tween	Flow through (% of total)	1M NaCl (% of total)	2% acetic acid (% of total)	6M GdnHCl (% of total)	Beads (% of total)
YYWFYY	No Tween	8.07	0.80	65.98	19.37	5.78
	0.05% Tween	75.16	8.88	11.81	2.25	1.90
YYWLHH	No Tween	10.40	0.68	75.21	10.32	3.39
	0.05% Tween	71.94	9.64	14.60	2.01	1.81

Table 11: Effect of salt in secondary screening

Buffer Peptide	PBS (% Bound)	PBS + 1M NaCl (% Bound)
Amino	8.15	10.93
Acetylated	4.77	3.47
YYWFYY	93.61	97.35
YYWLHH	93.43	97.54
YIWHHI	12.09	27.30
FYYLPE	4.74	4.77

Table 12: SEB binding sequences isolated from a phage-displayed library

No.	Sequence
1	**WHK**PPPSALGPK
2	**WHK**TPKATTQPL
3	**WHK**APRAPAPLL
4	**FHK**EWRPRPYAF
5	**WHR**PTPKPTLTI
6	**WHK**PPVRPPSTQ
7	**WHK**IPQKAPLNP
8	**WHK**QKPMTAPYP
9	**WHK**FPPRPPSLG

Figure 1: The 3D structure of SEB (Papageorgiou et al., 1998).

Figure 2: Split synthesis of the one-bead-one-peptide library. The resin beads are divided equally into separate reaction vessels each with a single amino acid. After the first amino acid is coupled to the resins, beads are repooled, mixed thoroughly, and redistributed into separate reaction vessels. The next coupling step is then performed. This divide-couple-recombine technique is repeated until the desired length of the peptide library is reached. There are X^n random sequences in the library, where X is the number of amino acids used for coupling, and n is the length of the library. Each resin bead displays only one peptide sequence.

Figure 3: The binding of SEB to different columns using PBS as binding buffer. 0.5mg/ml of SEB in a 100µl loop were injected into five different columns (amino resin, acetylated amino resin, YIWHHI, FYYLPE and YYWLHH resins), respectively. The samples in binding buffer (PBS) were injected at a flow rate of 0.02ml/min for 10mins. The flow rate was increased to 0.15ml/min for the remainder of the run. The column was washed sequentially with the binding buffer for 10mins, 1M NaCl in PBS for 20mins, and then 2% acetic acid in water for another 20mins.

Figure 4: The binding of SEB to different columns using 0.5M sodium chloride in PBS as binding buffer. 0.5mg/ml of SEB in a 100µl loop were injected into five different columns (amino resin, acetylated amino resin, YIWHHI, FYYLPE and YYWLHH resins), respectively. The samples in binding buffer (PBS + 0.5M NaCl) were injected at

a flow rate of 0.02ml/min for 10mins. The flow rate was increased to 0.15ml/min for the remainder of the run. The column was washed sequentially with the binding buffer for 10mins, 1M NaCl in PBS for 20mins, and then 2% acetic acid in water for another 20mins.

Figure 5: The binding of SEB to different columns using 1M sodium chloride in PBS as binding buffer. 0.5mg/ml of SEB in a 100µl loop were injected into five different columns (amino resin, acetylated amino resin, YIWHHI, FYYLPE and YYWLHH resins), respectively. The samples in binding buffer (PBS + 1M NaCl) were injected at a flow rate of 0.02ml/min for 10mins. The flow rate was increased to 0.15ml/min for the remainder of the run. The column was washed sequentially with the binding buffer for 10mins, 1M NaCl in PBS for 20mins, and then 2% acetic acid in water for another 20mins.

Figure 6: The effect of salt on SEB binding to a YYWLHH column. The following binding buffers with different sodium chloride concentrations were used: 10mM phosphorate buffer, pH 7.4, PBS, PBS + 0.1M NaCl, PBS + 0.25M NaCl, PBS + 0.5M NaCl, and PBS + 1.0M NaCl. 0.5mg/ml of SEB in binding buffer was injected at a flow rate of 0.02ml/min through a 100µl loop for 10mins. The flow rate was increased to 0.15ml/min for the remainder of the run. The column was washed sequentially with binding buffer for 10mins, 1M NaCl in PBS for 20mins, and then 2% acetic acid in water for another 20mins.

Figure 7: The effect of salt on the binding of *E. coli* lysate to a YYWLHH column. The following binding buffers with different sodium chloride concentrations were used: PBS, PBS + 0.25M NaCl, PBS + 0.5M NaCl, and PBS + 1.0M NaCl. 1mg/ml *E. coli* lysate in binding buffer was injected at a flow rate of 0.02ml/min through a 100µl loop for 10mins. The flow rate was increased to 0.15ml/min for the remainder of the run. The column was washed sequentially with binding buffer for 10mins, 1M NaCl in PBS for 35mins, and then 2% acetic acid in water for 20mins.

Figure 8: The effect of salt on the separation of SEB from *E. coli* lysate using a YYWLHH column. The following binding buffers with different sodium chloride concentrations were used: PBS, PBS + 0.25M NaCl, PBS + 0.5M NaCl, and PBS + 1.0M NaCl. 1mg/ml *E. coli* lysate with 0.5mg/ml SEB in binding buffer was injected at a flow rate of 0.02ml/min through a 100µl loop for 10mins. The flow rate was increased to 0.15ml/min for the remainder of the run. The column was washed sequentially with binding buffer for 10mins, 1M NaCl in PBS for 35mins, and then 2% acetic acid in water for 20mins.

Figure 9: The effect of salt on the binding of *E. coli* lysate to an amino column. The following binding buffers with different sodium chloride concentrations were used: PBS, PBS + 0.25M NaCl, PBS + 0.5M NaCl, and PBS + 1.0M NaCl. 1mg/ml *E. coli* lysate in binding buffer was injected at a flow rate of 0.02ml/min through a 100µl loop for 10mins. The flow rate was increased to 0.15ml/min for the remainder of the run. The column was

washed sequentially with binding buffer for 10mins, 1M NaCl in PBS for 20mins, and then 2% acetic acid in water for another 20mins.

Figure 10: The effect of salt on the binding of *E. coli* lysate to an acetylated amino column. The following binding buffers with different sodium chloride concentrations were used: PBS, PBS + 0.25M NaCl, PBS + 0.5M NaCl, and PBS + 1.0M NaCl. 1mg/ml *E. coli* lysate in binding buffer was injected at a flow rate of 0.02ml/min through a 100µl loop for 10mins. The flow rate was increased to 0.15ml/min for the remainder of the run. The column was washed sequentially with binding buffer for 10mins, 1M NaCl in PBS for 20mins, and then 2% acetic acid in water for another 20mins.

Figure 11: Purification of SEB from *E. coli* lysate using a YYWLHH column. The chromatograms represent the injection of 1mg *E. coli* lysate with increasing quantities of SEB on the peptide column YYWLHH. The samples in binding buffer (PBS + 0.5M NaCl) were injected at a flow rate 0.02ml/min through a 100µl loop for 10mins. The flow rate was increased to 0.15ml/min for the remainder of the run. The column was washed sequentially with binding buffer for 10mins, 1*M* NaCl in PBS for 20mins, and then 2% acetic acid in water for another 20mins.

Figure 12: SDS-PAGE of the fractions from chromatography runs of the mixture of 1mg/ml *E. coli* lysate and 0.5mg/ml SEB using a YYWLHH column. M, E, S, and I denote the molecular weight markers*, E. coli* lysate, SEB standard, and injected sample.

Lane 1 and 2 correspond to the peaks of flow through and 1M NaCl. Lane 3 and 4 correspond to the first peak and second peak of 2% acetic acid.

Figure 13: The effect of concentrations of *E. coli* lysate on the recovery of SEB using a YYWLHH column. The chromatograms represent the injection of 0.5mg SEB with increasing quantities of *E. coli* lysate on the peptide column YYWLHH. The samples in binding buffer (PBS + 0.5M NaCl) were injected at a flow rate 0.02ml/min through a 100µl loop for 10mins. The flow rate was increased to 0.15ml/min for the remainder of the run. The column was washed sequentially with binding buffer for 10mins, 1M NaCl in PBS for 20mins, and then 2% acetic acid in water for another 20mins.

Figure 14: SDS-PAGE of the fractions from chromatography runs of the mixture of 5mg/ml *E. coli* lysate and 0.5mg/ml SEB using a YYWLHH column. M, E, S, and I denote the molecular weight markers, *E. coli* lysate, SEB standard, and injected sample. Lane 1 and 2 correspond to the peaks of flow through and 1M NaCl. Lane 3 and 4 correspond to the first peak and second peak of 2% acetic acid.

Figure 15: Purification of SEB from BSA using a YYWLHH column. The chromatograms represent the injection of 5mg BSA with increasing quantities of SEB on the peptide column YYWLHH. The samples in binding buffer (PBS + 0.5M NaCl) were injected at a flow rate 0.02ml/min through a 100µl loop for 10mins. The flow rate was increased to 0.15ml/min for the remainder of the run. The column was washed

sequentially with binding buffer for 10mins, 1M NaCl in PBS for 20mins, and then 2% acetic acid in water for another 20mins.

Figure 16: SDS-PAGE of the fractions from chromatography runs of the mixture of 5mg/ml BSA and 0.5mg/ml SEB using a YYWLHH column. M, B, and S, denote the molecular weight markers, BSA, and SEB standard. Lane 1 corresponds to the peak of flow through. Lane 2 and 3 correspond to the first peak and second peak of 2% acetic acid.

Figure 17: The binding of BSA to control columns. 5mg/ml BSA was loaded on an amino column and an acetylated amino column, respectively. The samples in binding buffer (PBS + 0.5M NaCl) were injected at a flow rate 0.02ml/min through a 100µl loop for 10mins. The flow rate was increased to 0.15ml/min for the remainder of the run. The column was washed sequentially with binding buffer for 10mins, 1M NaCl in PBS for 20mins, and then 2% acetic acid in water for another 20mins.

DOMAIN 2 DOMAIN 1

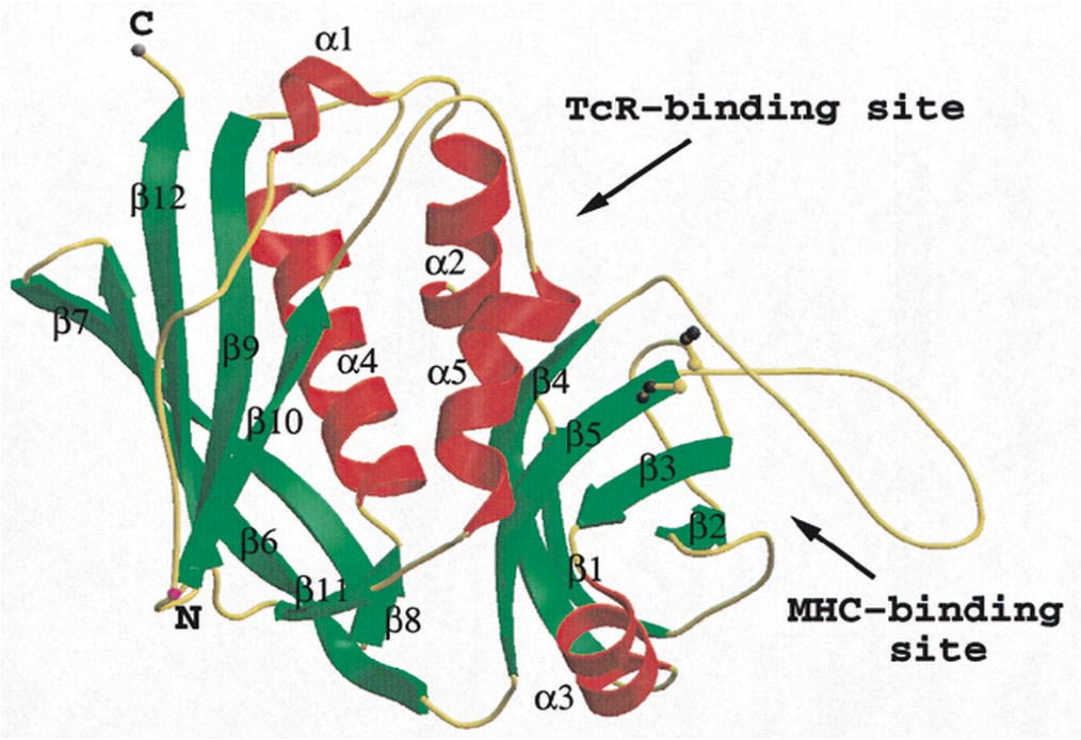


Figure 1: The 3D structure of SEB

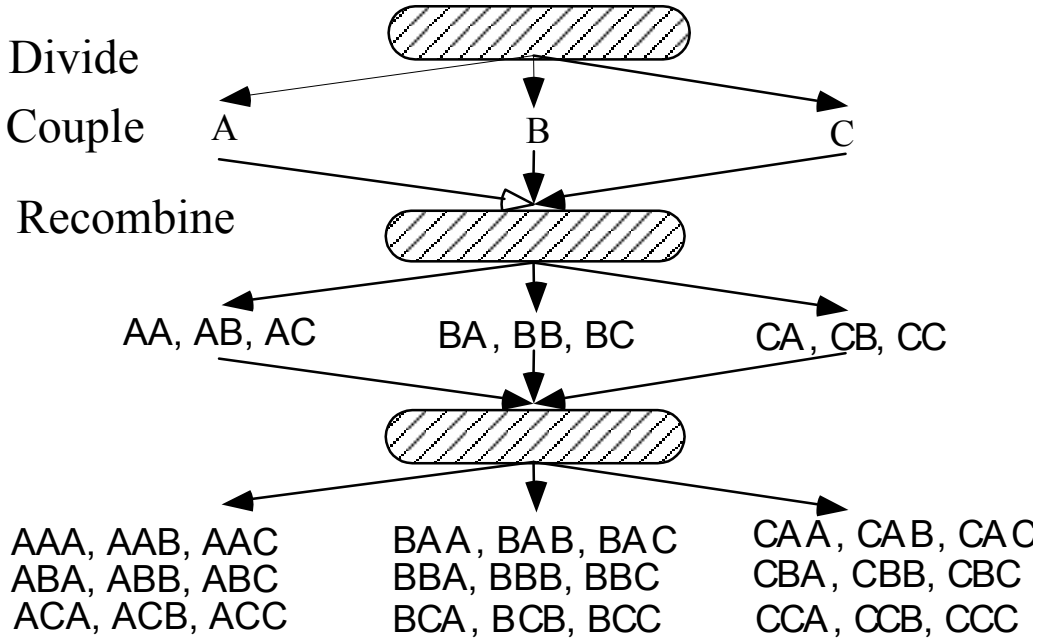


Figure 2: Split synthesis of the one-bead-one-peptide library

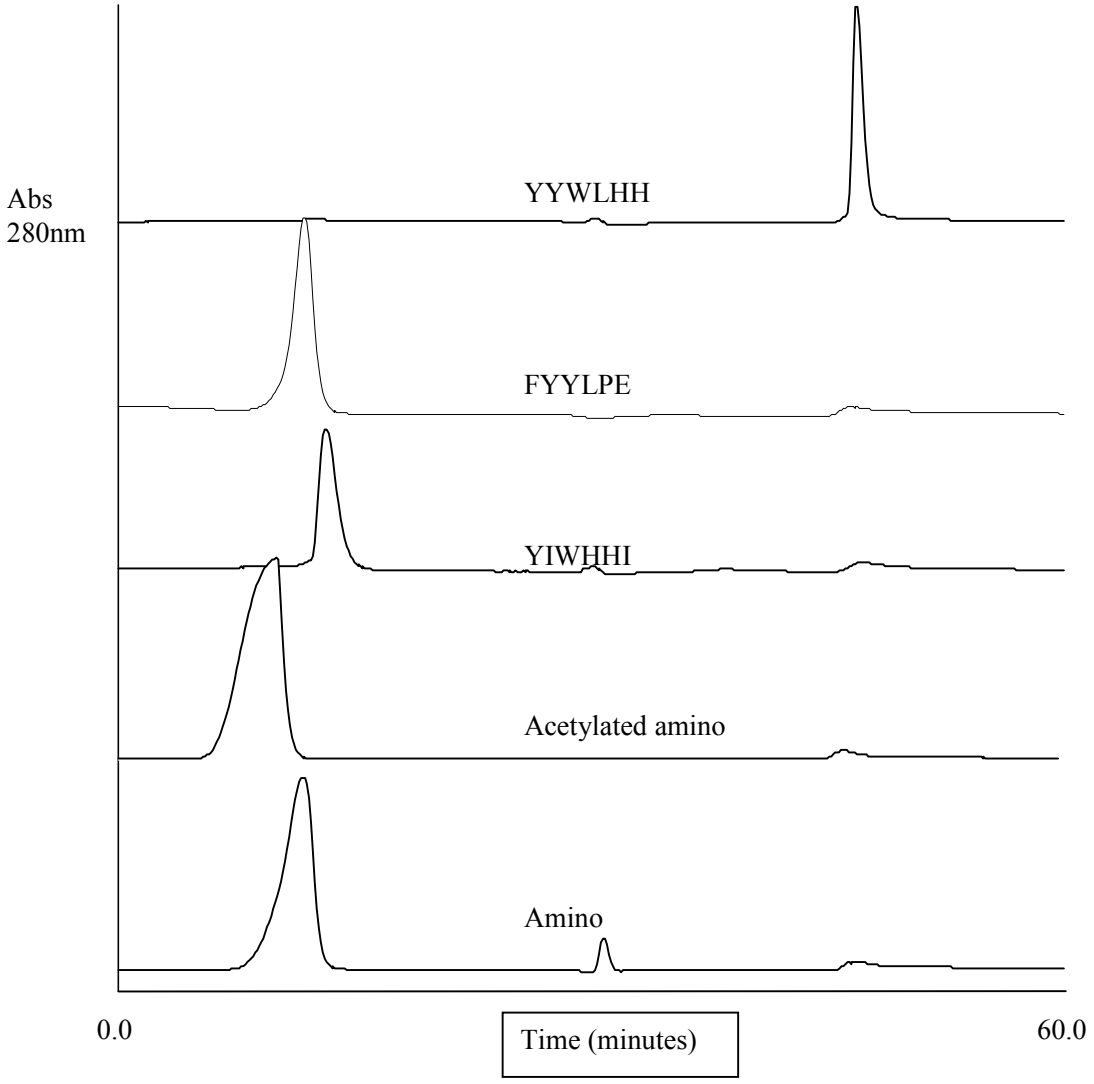


Figure 3: The binding of SEB to different columns using PBS as binding buffer.

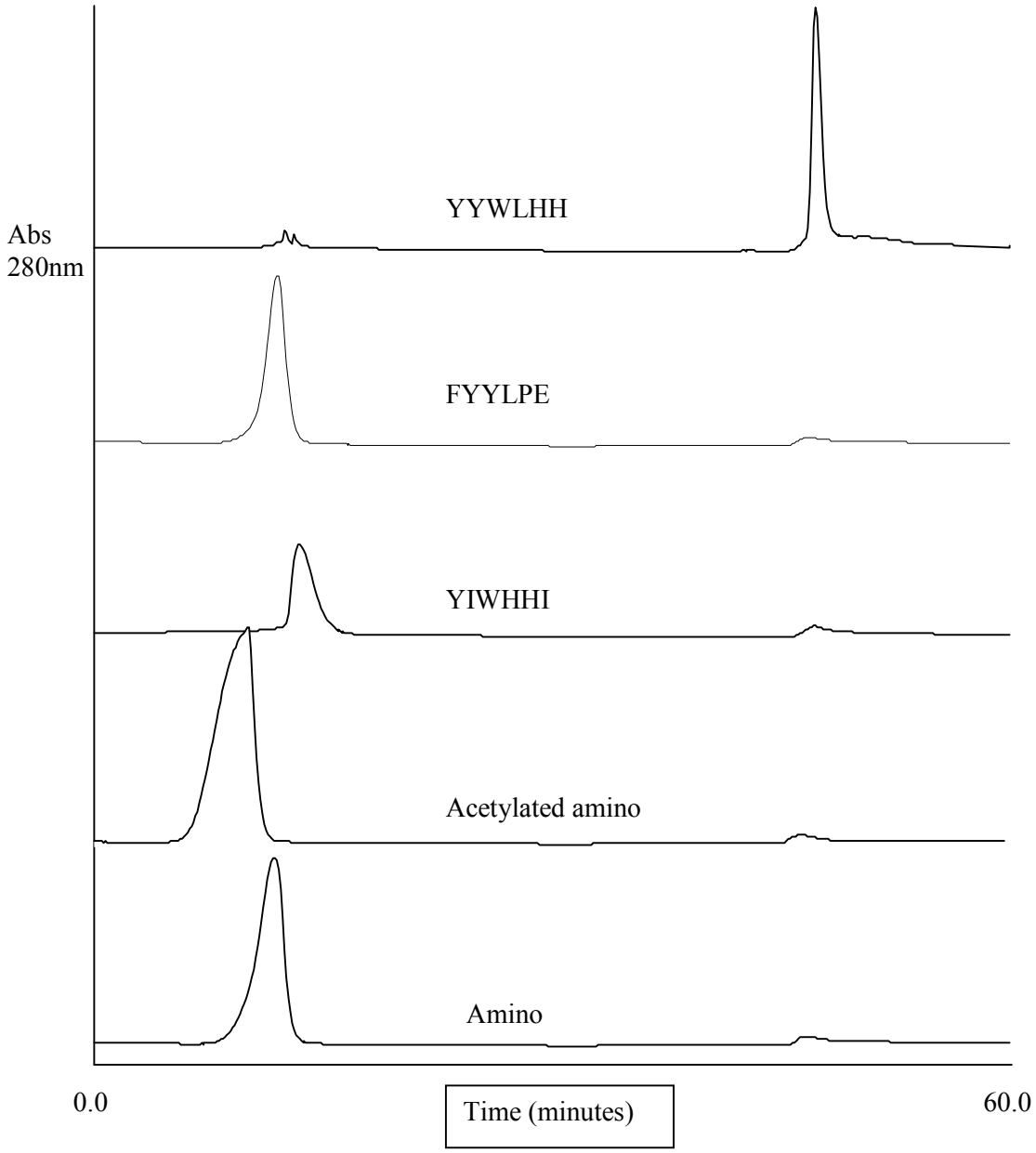


Figure 4: The binding of SEB to different columns using 0.5M sodium chloride in PBS as binding buffer.

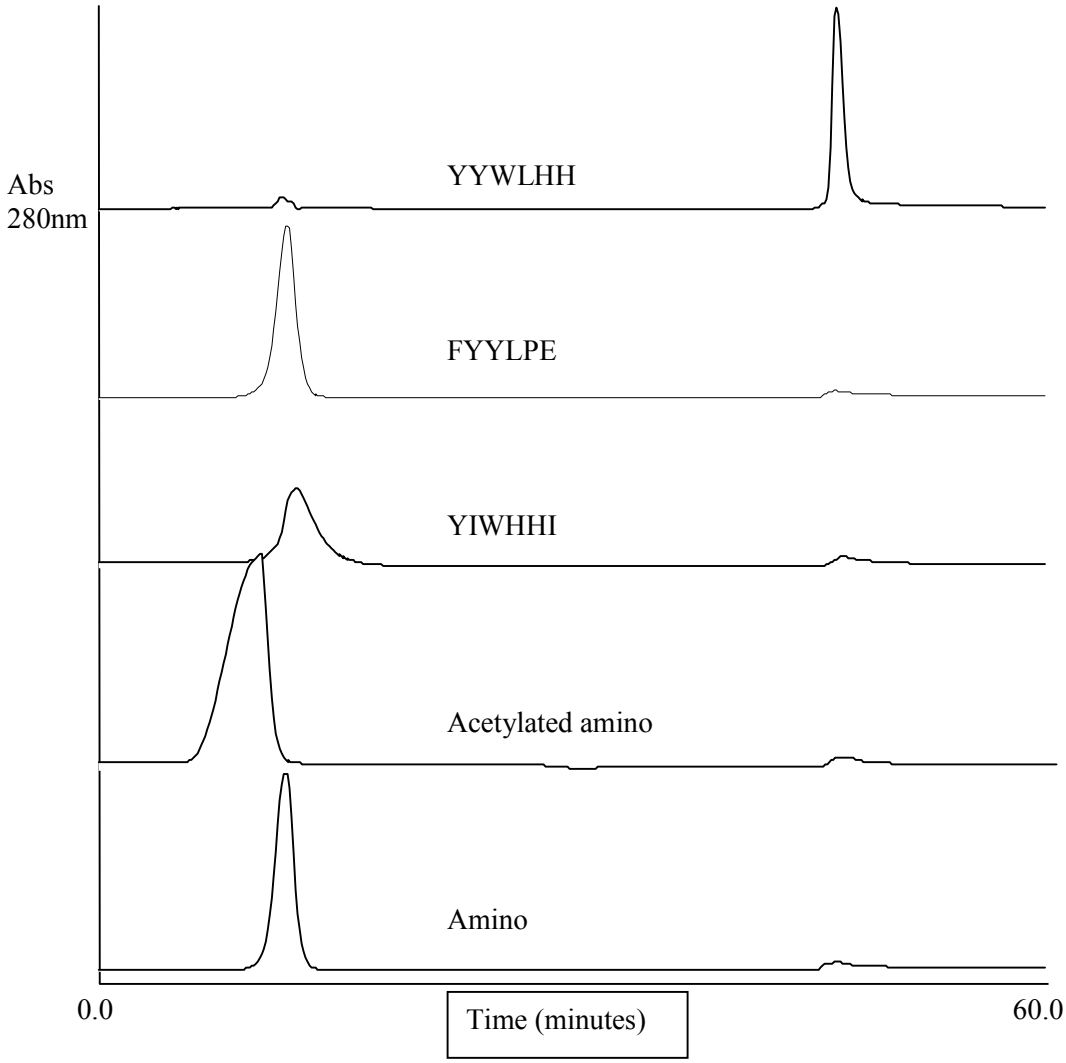


Figure 5: The binding of SEB to different columns using 1.0 M sodium chloride in PBS as binding buffer.

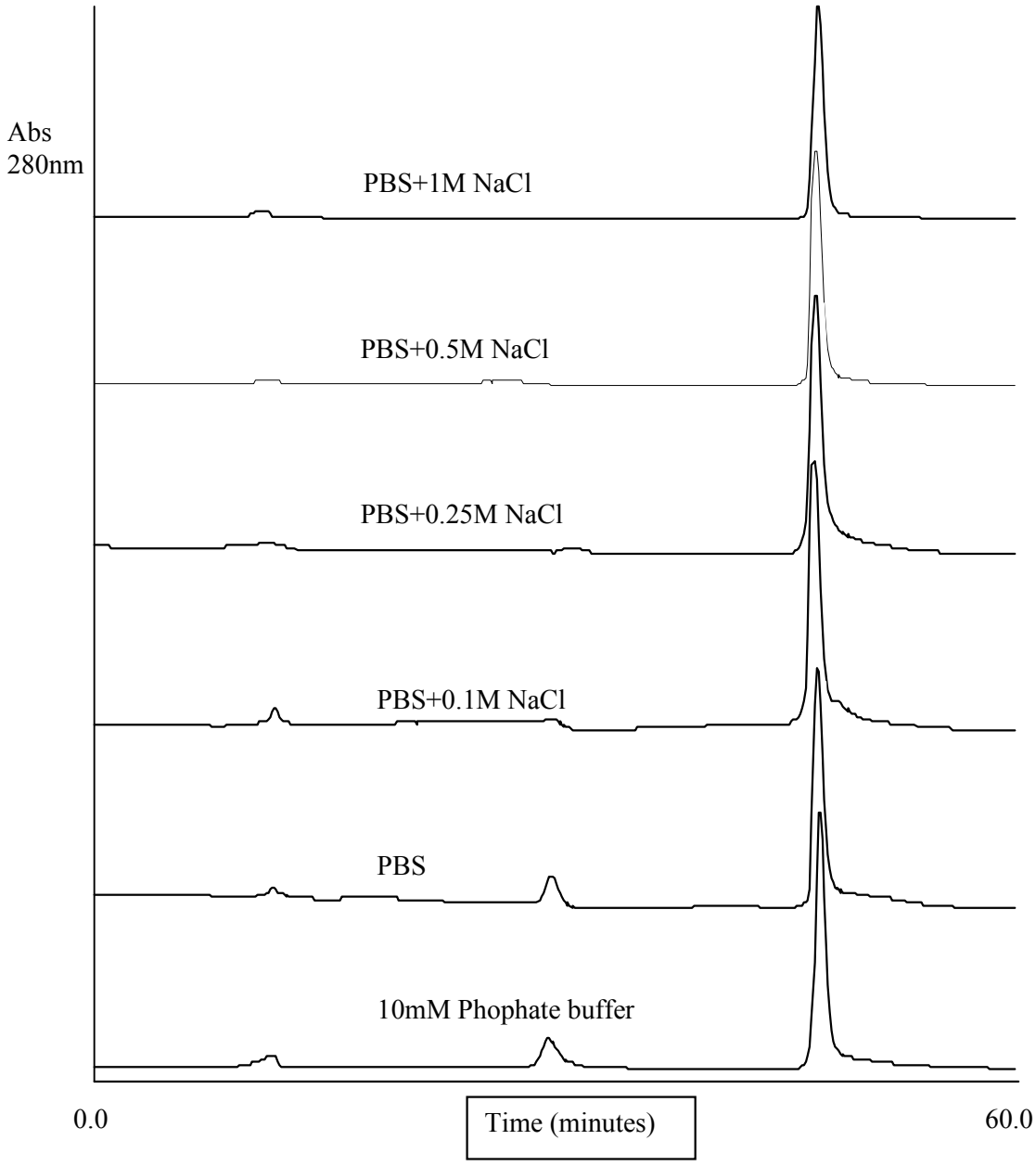


Figure 6: The effect of salt on SEB binding to a YYWLHH column.

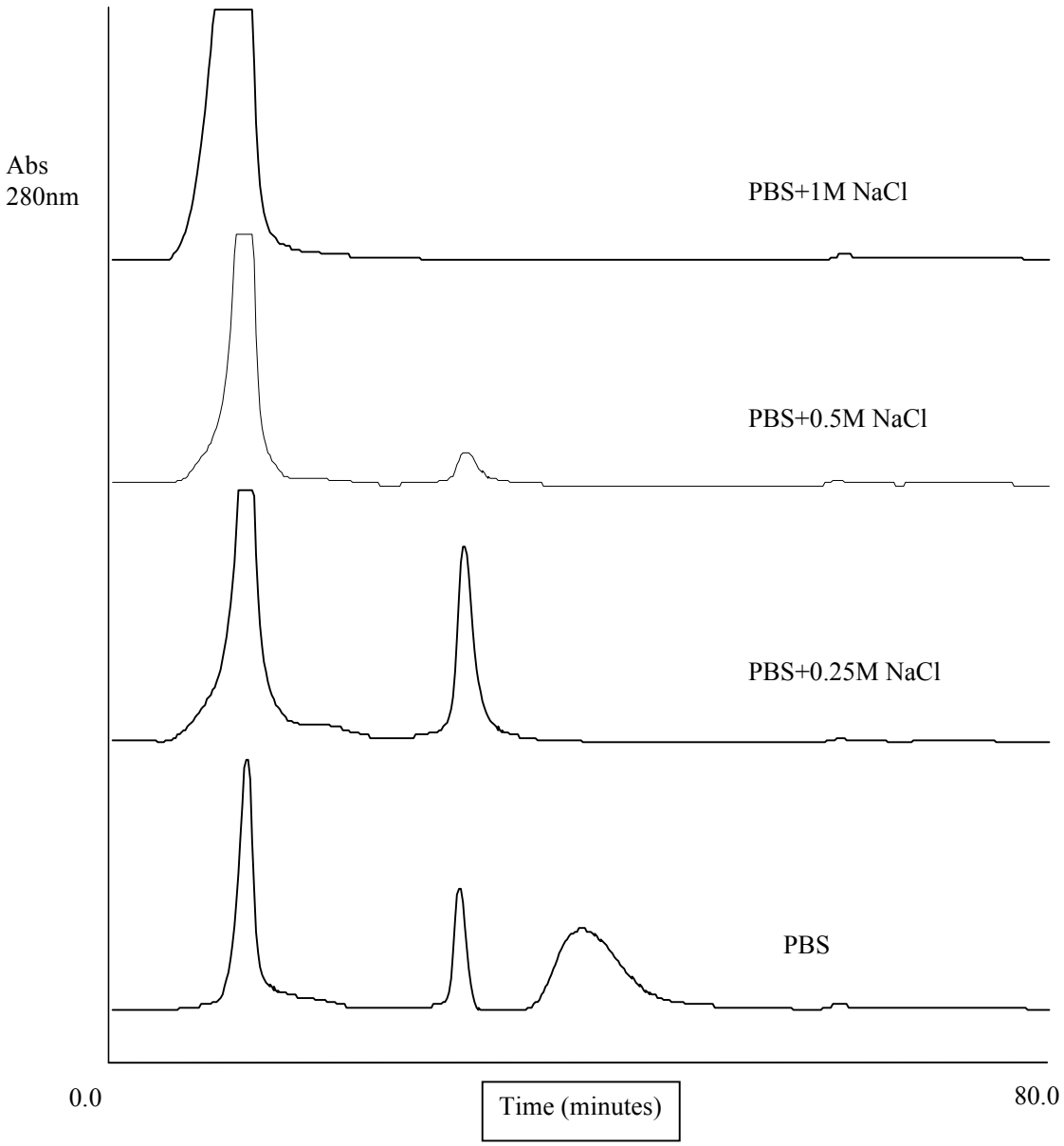


Figure 7: The effect of salt on the binding of *E. coli* lysate to a YYWLHH column.

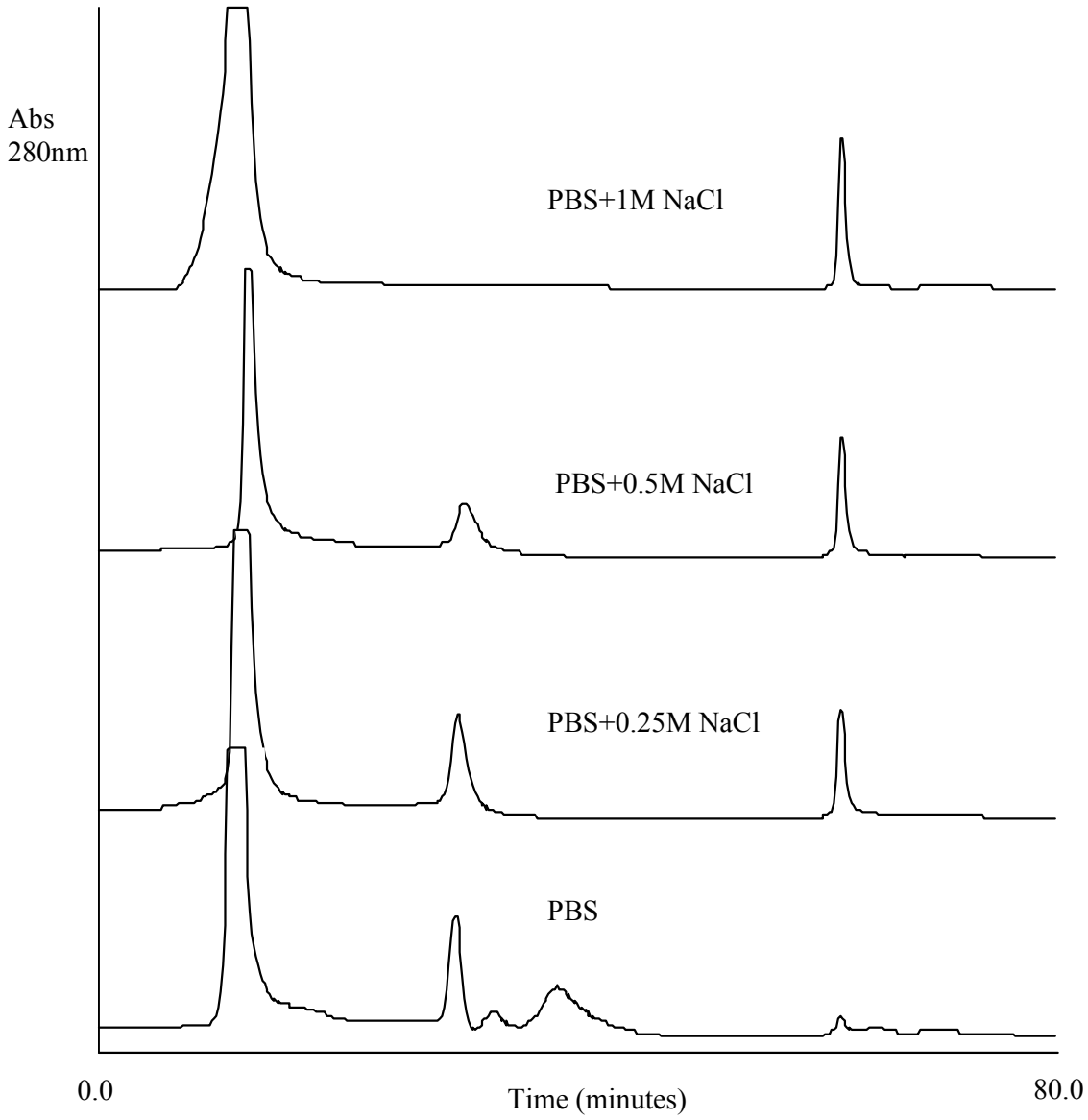


Figure 8: The effect of salt on the separation of SEB from *E. coli* lysate using a YYWLHH column.

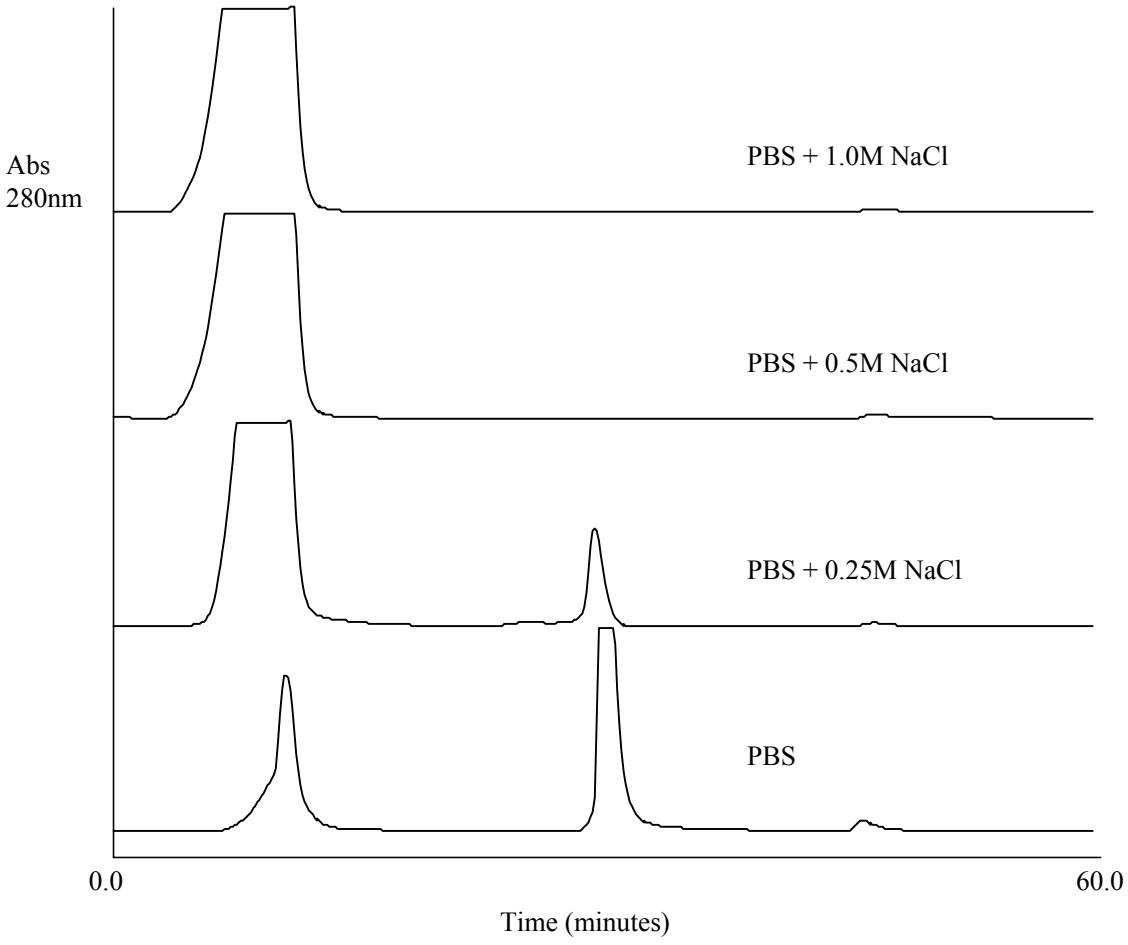


Figure 9: The effect of salt on the binding of *E. coli* lysate to an amino column.

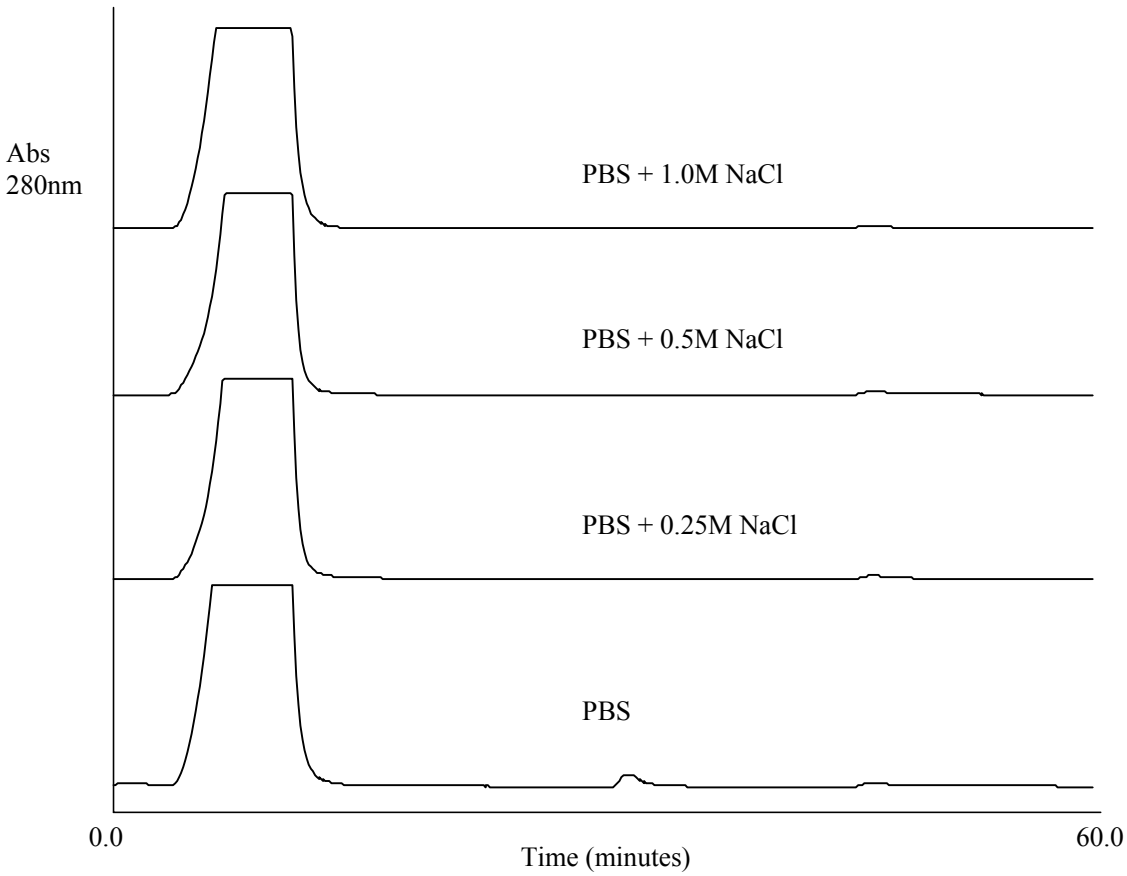


Figure 10: The effect of salt on the binding of *E. coli* lysate to an acetylated amino column.

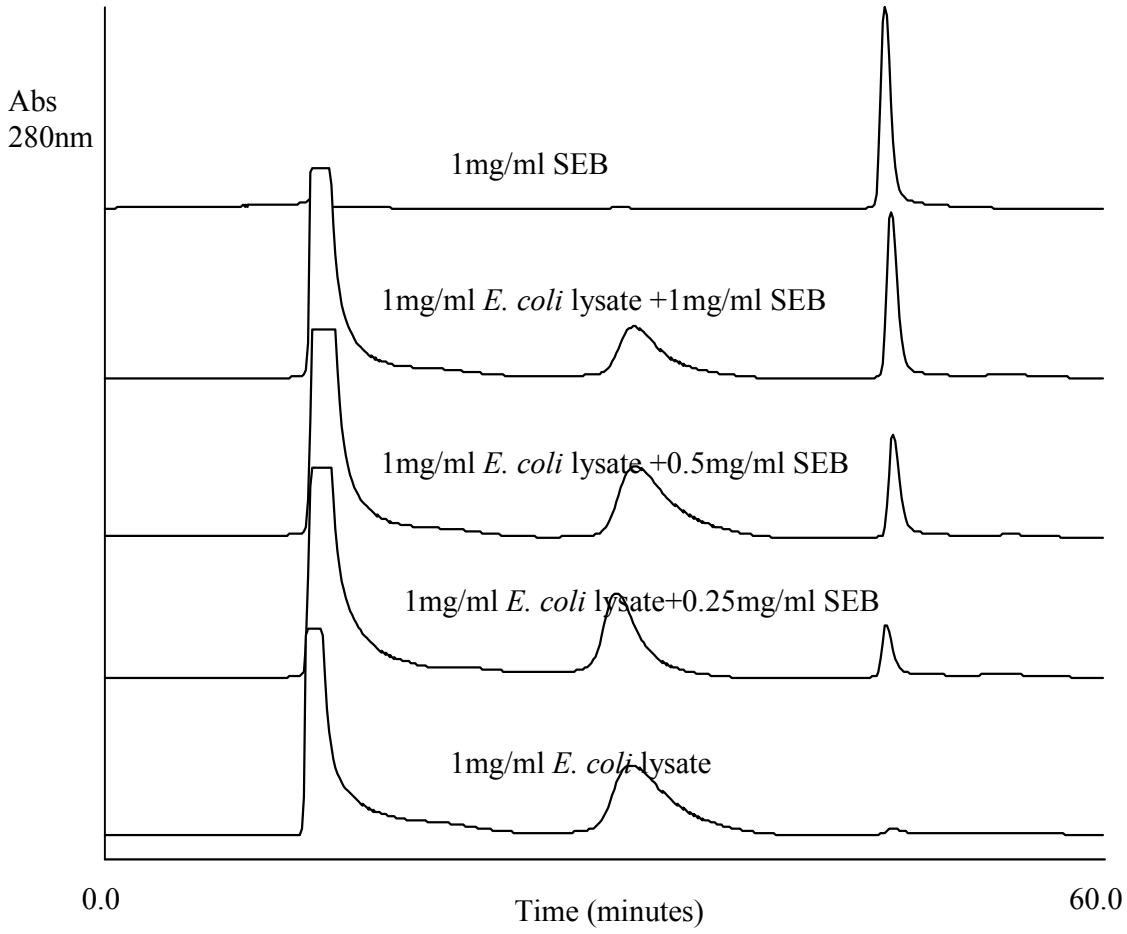


Figure 11: Purification of SEB from *E. coli* lysate using a YYWLHH column.

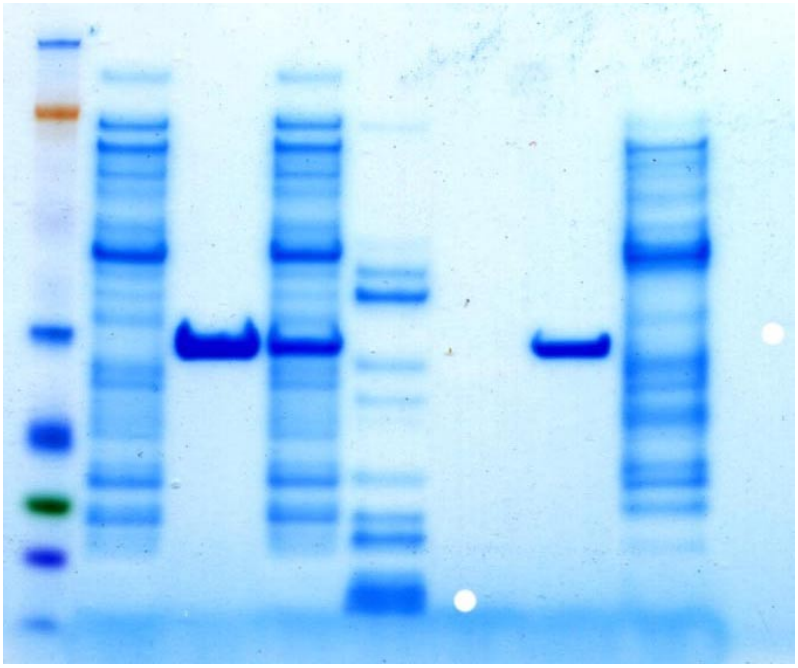


Figure 12: SDS-PAGE of the fractions from chromatography runs of the mixture of 1mg/ml *E. coli* lysate and 0.5mg/ml SEB using a YYWLHH column.

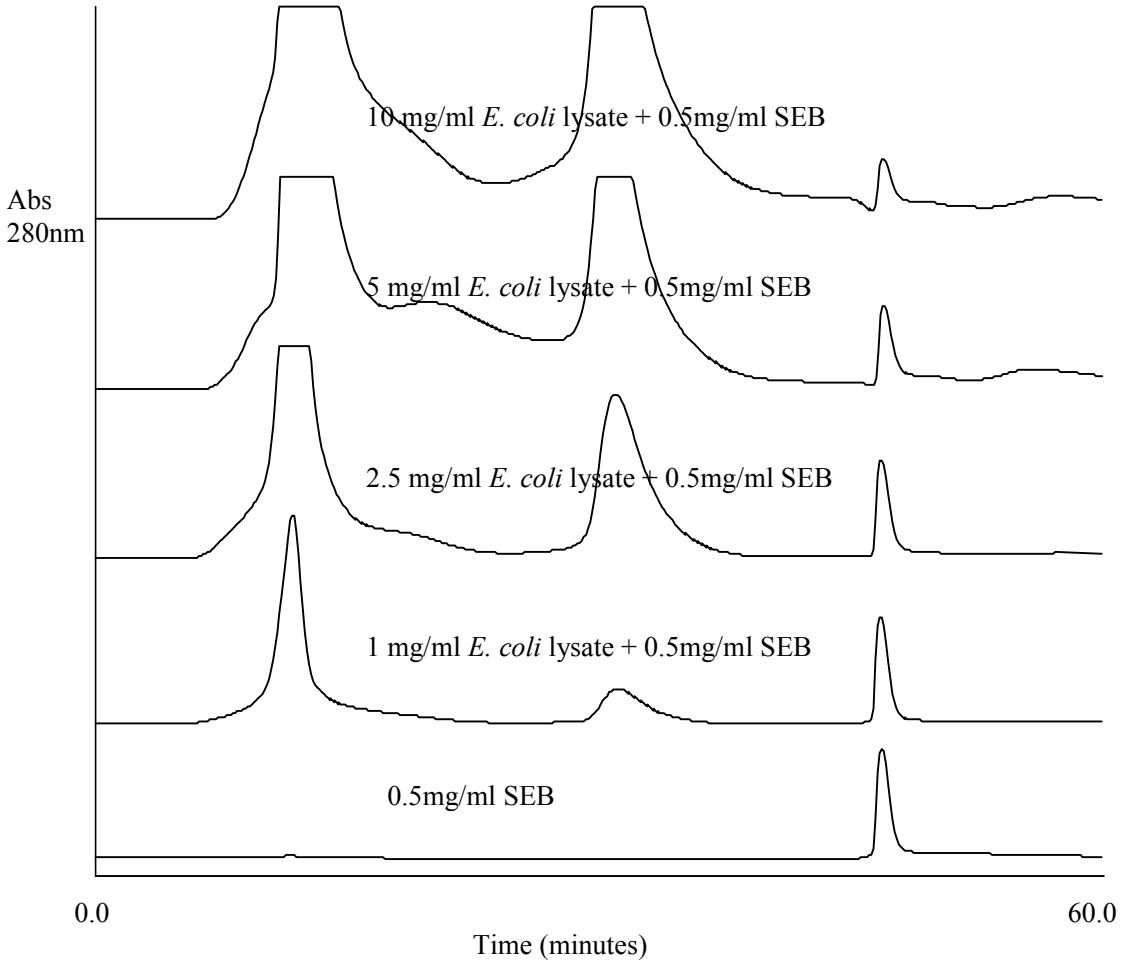
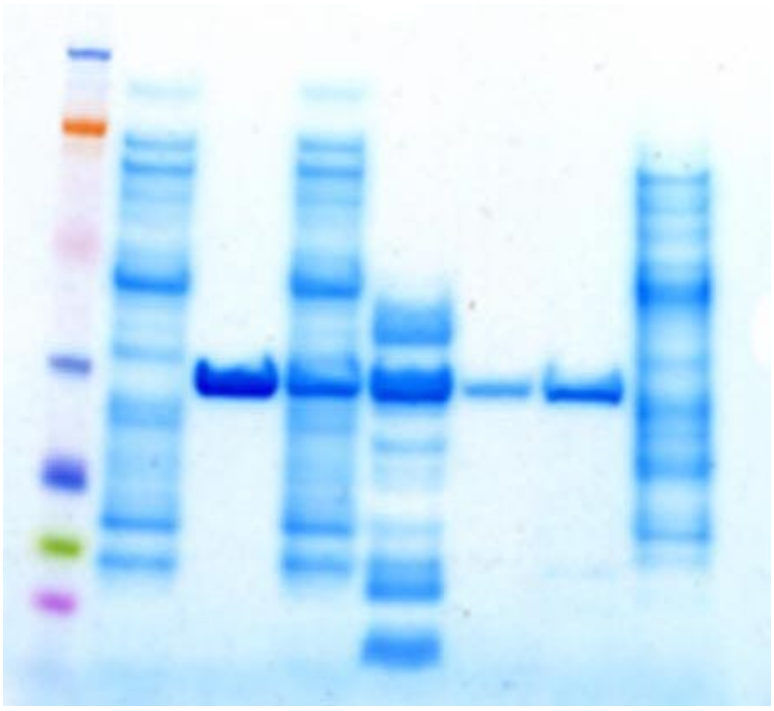


Figure 13: The effect of concentrations of *E. coli* lysate on the recovery of SEB using a YYWLHH column



M E T I 1 2 3 4

Figure 14: SDS-PAGE of the fractions from chromatography runs of the mixture of 5mg/ml *E. coli* lysate and 0.5mg/ml SEB using a YYWLHH column.

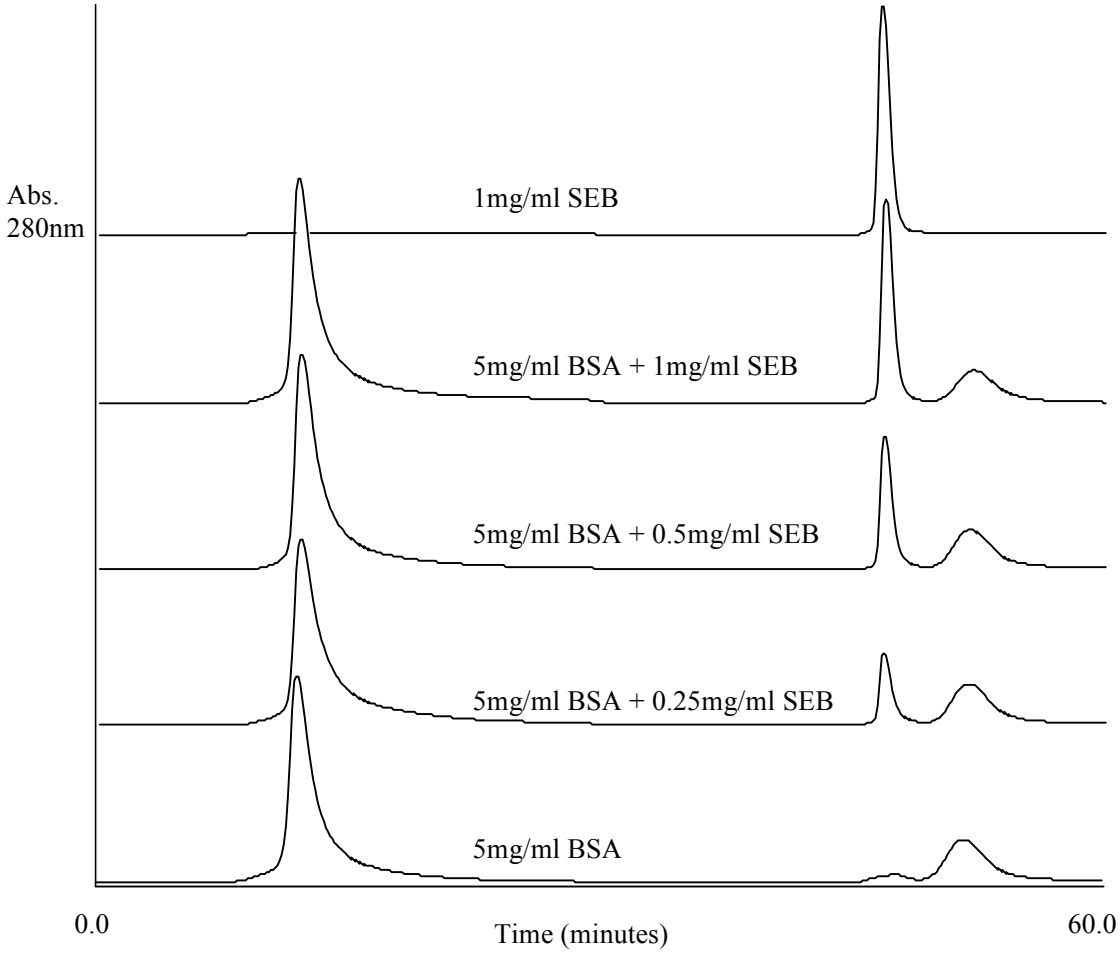


Figure 15: Purification of SEB from BSA using a YYWLHH column.

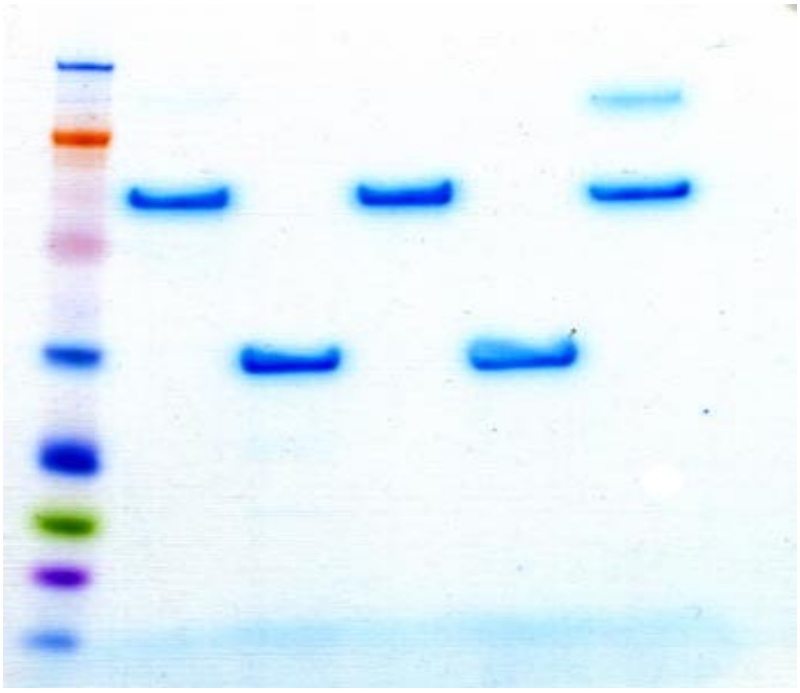


Figure 16: SDS-PAGE of the fractions from chromatography runs of the mixture of 5mg/ml BSA and 0.5mg/ml SEB using a YYWLHH column.

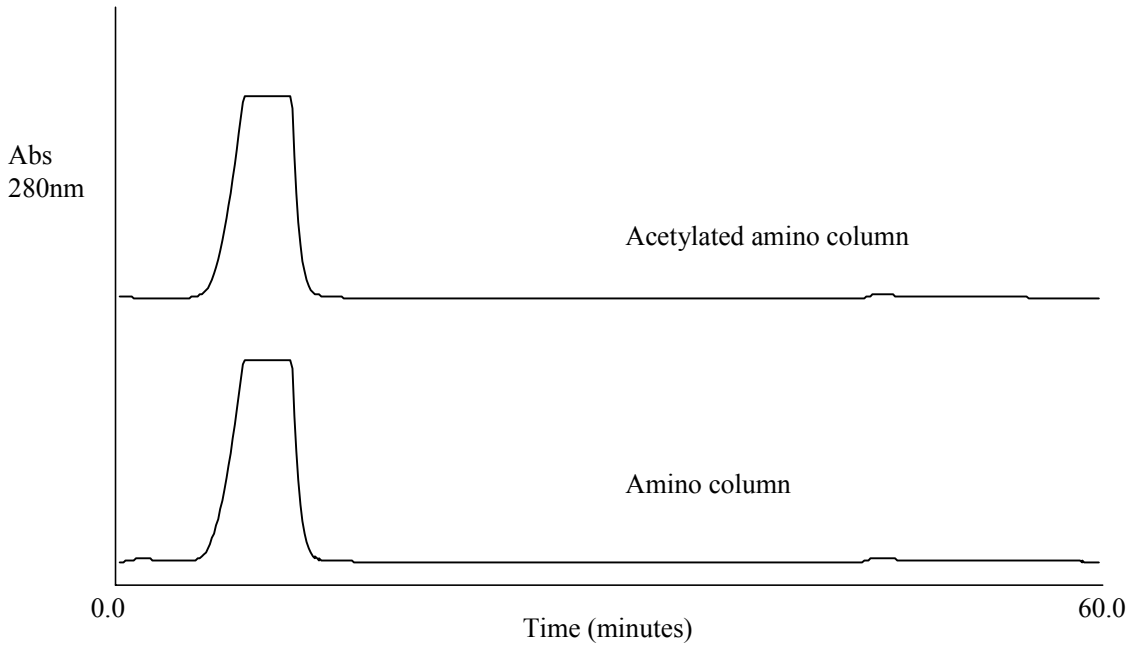


Figure 17: The binding of BSA to control columns