

Research article

Enhanced immunogenicity of a functional enzyme by T cell epitope modification

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Abstract

Background: T helper epitopes are necessary for the induction of high titers of antigen-specific IgG antibodies. We are interested in the epitope modification of intact proteins as a method to enhance their immunogenicity for the generation of recombinant protein-based vaccines.

Results: Hartley strain guinea pig T cell epitopes were mapped for two related bacterial proteases. Two T cell epitopes were found in one of the proteases, while a comparatively reduced immunogenicity protease had no detectable T cell epitopes. A T cell epitope sequence homologous to the immunogenic protease was created in the less immunogenic protease by changing a single amino acid. Proliferative responses to the whole protein parent enzyme were two-fold higher in splenocyte cultures from variant-immunized animals. We found that the single amino acid change in the variant resulted in a protein immunogen that induced higher titers of antigen-specific IgG antibody at low doses and at early time points during the immunization protocol. The serum from parent- and variant-immunized guinea pigs cross-reacted at both the protein and the peptide level. Finally, animals primed to the variant but boosted with the parent enzyme had higher levels of antigen-specific IgG than animals immunized with the parent enzyme alone.

Conclusions: With a single amino acid change we have introduced a T cell epitope into a comparatively low-immunogenic enzyme and have increased its immunogenicity while retaining the enzyme's original proteolytic function. The ability to immunomodulate proteins while leaving their function intact has important implication for the development of recombinant vaccines and protein-based therapeutics.

Background

High affinity humoral immune responses to most protein antigens require cognate interactions between antigen-specific T and B cells. Antigen-specific T cells encounter antigen presented by dendritic cells that migrate to the

paracortical regions of draining lymph nodes after initial antigen contact [1]. Only dendritic cells have the capacity to induce activation in resting peripheral T cells [2,3]. Once activated, differentiated T helper cells contact antigen-specific B cells and provide signals for B cell differen-

tiation via CD154-CD40 interactions, as well as by the production of cytokines [4–6]. Consistent with this general understanding of the induction of antibody responses to protein immunogens, good antibody responses to synthetic peptide-epitope constructs have been shown to depend on the presence, orientation, and multiplicity of the T cell epitope in the construct [7–11]. As a confirmation of this finding with synthetic epitope constructs, most protein immunogens studied to date contain multiple T cell epitopes, for example [12,13].

T helper epitopes have become common components of synthetic vaccine constructs due to their ability to provide immunological help for both humoral and cell-mediated responses [11,14–16]. T cell epitopes that can induce helper activity by binding to a wide range of HLA-DR alleles have been developed by a number of investigators using various antigen systems [17–19]. T cell epitopes that have the property of binding across a wide range of HLA-DR haplotypes are necessary for the construction of synthetic vaccines that would be useful in the human population as a whole. DNA- and peptide-based vaccines have become popular due to the comparative ease of construction, the circumvention of potential safety issues around attenuated organisms, and for their potentially enhanced immunogenicity compared to heat-killed and subunit vaccines [20–22]. DNA vaccines have the added attraction of efficiently priming both humoral and cytotoxic cell responses, a property largely lacking in subunit and attenuated organism vaccines. Priming of cytotoxic cell responses is necessary for the design of therapeutic cancer vaccines, as well as for viral vaccines. Multiple antigen peptide vaccines can also be designed to contain T and B epitope regions from numerous protein antigens derived from a complex infectious agent which could therefore confer broad protection without the potential dangers of an attenuated whole organism vaccine [16]. However, it is of note that occasionally a well-designed peptide-based vaccine can elicit strong antibody responses to the synthetic immunogen that do not confer protection from the parasite to which they were originally designed [23]. This may be due to either the induction of an inappropriate antibody isotype in the mouse strain used, or to an inadequate overall response.

While subunit vaccines have their inherent shortcomings, they carry the added benefit of potentially inducing complex antibody responses to multiple sites on the whole protein immunogen. Subunit vaccines are of interest in the prevention of parasite infection [24] and for a variety of infectious diseases [25]. An ideal subunit vaccine for the induction of protective antibody responses would be a protein from the disease-causing organism that was minimally modified from its original structure to retain a variety of potential B cell epitopes. In addition, the modi-

fied subunit vaccine would be designed to be highly immunogenic by the addition of promiscuous T cell epitopes. To this end, we have characterized the immune response of partially outbred Hartley strain guinea pigs to two related bacterial proteases. The two bacterial enzymes studied represent important industrial enzymes used in a number of applications, including serving as an additive for laundry products, and animal feed processing. These enzymes have been well characterized as to their activity and specificity by us and by others [26,27]. While these two proteins are approximately 60% homologous at the amino acid sequence [26], one of the proteases was significantly less immunogenic than the other. Reduced immunogenicity was characterized as significantly lower titers of antigen-specific IgG when animals were immunized with the same amount of the enzymes. We determined the T cell epitopes in both of the proteases using 15-mer peptides and splenocytes from immunized animals. In order to be classified as an epitope, the average proliferative response to a peptide had to reach a stimulation index of 3.0. Two regions met our criteria as epitopes in the immunogenic protease. We tested a total of 20 guinea pigs and found no epitope regions in the less immunogenic protease. A sequence comparison of the enzymes in the T cell epitope regions revealed that a single amino acid change could confer a T cell epitope onto the less-immunogenic protease. This variant was constructed, and was found to be a functional and stable protease. Immunization of Hartley strain guinea pigs showed that this single amino acid change created a T cell epitope where there was no detectable epitope before, and the presence of this epitope correlates with an improved immunogenicity in the variant. Finally, we show that the variant induces broadly cross-reactive IgG antibodies, and that priming of animals with the variant protein results in higher titers of antigen-specific antibody upon subsequent immunization with the parent enzyme.

Results

IgG antibody titers from *B. lentus subtilisin* and BPN' Y217L guinea pig immunizations

Female Hartley strain guinea pigs were immunized with two related bacterial proteases, *B. lentus* subtilisin and BPN' Y217L. These two bacterial enzymes share 60% homology at the amino acid level. They are also comparable in their proteolytic activity and specificity [26,27]. Animals were immunized every two weeks with a 20 ug dose of enzyme in adjuvant. Serum samples were drawn prior to each immunization over a ten-week period. Serum samples were tested for antigen-specific IgG antibodies by ELISA. Titers were calculated for each animal's antibody response. Averages for four animals are presented in Figure 1. The titers over time are presented as serum dilution (B) and as Log₂ titers (A). Immunization with *B. lentus* subtilisin induced higher titers of antigen-specific IgG

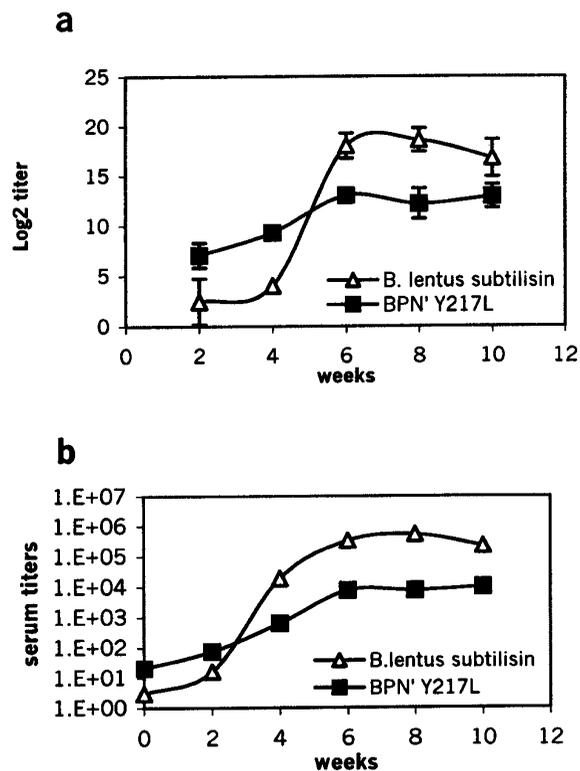


Figure 1

Guinea pig antigen-specific IgG responses to *B. lentus subtilisin* and BPN' Y217L. Two groups of four Hartley Guinea pigs were immunized with 20 µg of protease five times over a 10-week period. Serum samples were taken every two weeks. Antibody titers were determined by ELISA. Titers were calculated by determining the ODs at 405 nm where $\gamma = 0.5$, using a regression analysis of titer data for each animal. Figure 1A shows the average titers and standard deviations in a log base 2 format, Figure 1B represents the same data in a base 10 format.

than BPN' Y217L after 4 weeks. The response to *B. lentus subtilisin* reached maximal levels by about 6 weeks. At the 6 to 10 week time points, titers leveled off and the average titers induced by immunizations with *B. lentus subtilisin* were about 10 fold higher than BPN' Y217L. *B. lentus subtilisin* remained more immunogenic at doses from 1 to 20 µg. (not shown). Overall maximum titer levels vary, but the difference in immunogenicity between *B. lentus subtilisin* and BPN' Y217L has been seen in three separate guinea pig studies. This difference in immunogenicity has been noted by others using an intratracheal method of immunization [28,29].

T cell epitope mapping of *B. lentus subtilisin* and BPN' Y217L

Splenocytes from immunized guinea pigs were used to determine the position of T cell epitopes in *B. lentus subtilisin* and BPN' Y217L. 10 animals were tested for each immunogen. 15-mer peptides offset by 3 amino acids were used at 5 µM, and were tested at least in duplicate for each immunized animal. Peptides eliciting a 3-fold or higher average proliferative response over the background response were considered T cell epitopes. These epitopes are shown in bold in Figure 2. Two T cell epitopes were found in 5 of the 10 guinea pigs immunized with *B. lentus subtilisin*, but none meeting the criteria of a three-fold increase in proliferation over the background were found in the 10 animals immunized with BPN' Y217L in this experiment ($p = 0.01$; non-parametric Student' t-test). Notably, the first *B. lentus subtilisin* epitope has an analogous region in BPN' Y217L. only the first amino acid is different. It was hypothesized that if the first valine in the analogous BPN' Y217L sequence was changed to an isoleucine, a T cell epitope analogous to the one found in *B. lentus subtilisin* would be created. A modified BPN' Y217L V72I enzyme (GP002) was prepared with this single amino acid change (Figure 3).

Enzyme activity and stability

Enzymatic activity has been implicated as a source of the allergenic property of Der p1, the major dust mite allergen [30–32]. In order to account for this variable, the enzymatic activity of the variant protease GP002 was compared BPN' Y217L. Proteolytic activity was determined in a standard sAAPF hydrolysis assay. The activity of GP002 was found to be approximately 80% of the parent BPN' Y217L enzyme (Figure 4). Another factor that could influence the immunogenicity of a proteolytic enzyme is the relative stability of the protease. If the protease remains active for a longer period of time, any effects of the activity would be comparatively magnified. The half-lives of both enzymes were tested at 37°C over a 74-hour time period to assess relative stability. The assay was performed at physiologic pH = 7.3. At various time points, the proteolytic activity remaining in the samples were tested. At 37 degrees and a pH of 7.3, BPN' Y217L has a half-life fifty-five hours longer than GP002. These results indicate that the two enzymes have comparable stability and efficacy. Comparable enzymatic activity and stability suggest that the variant molecule has a similar tertiary configuration as the parent.

T-cell epitope mapping BPN' Y217L versus GP002

Hartley strain guinea pigs were immunized with 5 µg per dose of either BPN' Y217L or GP002 every two weeks. After 6 weeks epitope mapping was performed as described using the BPN'Y217L 15-mer PepSet and splenocytes from the primed animals (Figure 5). In addition, prolifer-

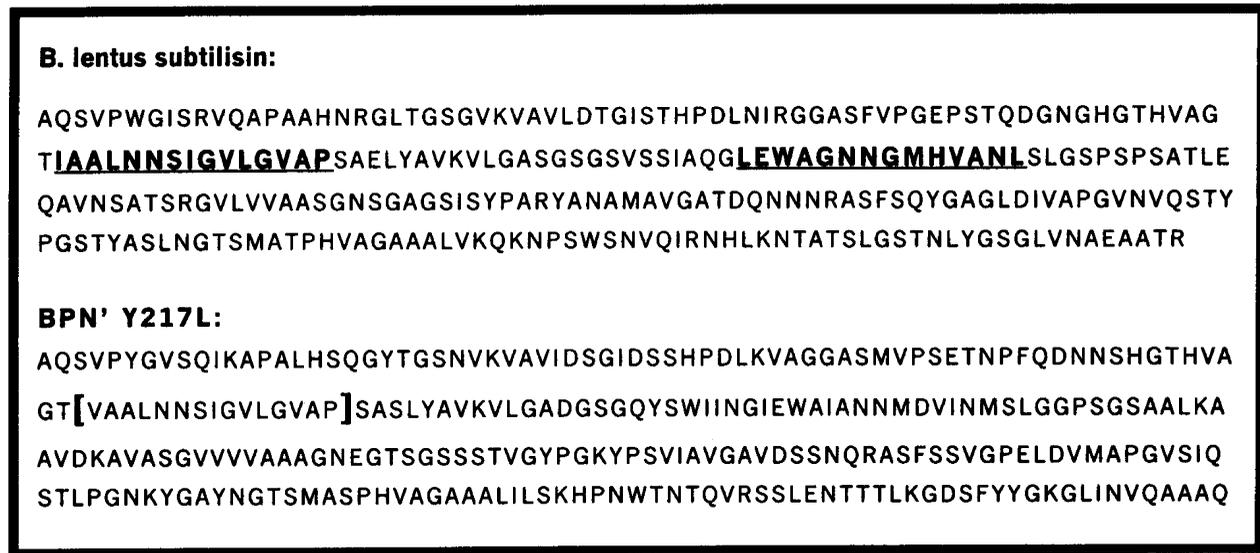


Figure 2

Guinea pig T cell epitopes in *B. lentus* subtilisin and BPN' Y217L. Hartley strain guinea pigs were immunized with either *B. lentus* subtilisin or BPN' Y217L. Splenocytes from immunized Hartley Guinea pigs were incubated in vitro with 15 mer peptides describing the sequence of the immunizing protein as described in the material and methods section. An epitope region was assigned if the T cell proliferation to a peptide reached an average SI of 3.0. Two major T cell epitopes were found in *B. lentus* subtilisin (underlined). No T cell epitopes meeting our criteria were found in BPN' Y217L. The first *B. lentus* subtilisin epitope has a very similar region in BPN' Y217L (in brackets) which contains the single amino acid change of I 72 V.

ative responses to the intact inactivated BPN' Y217L enzyme were tested. Immunization with GP002 resulted in an increased proliferative response to whole protein BPN'Y217L. The average stimulation index (proliferative response to enzyme/proliferation in control cultures) to 20 ug of whole protein was 23, versus an average stimulation index of 11 for the BPN' Y217L-immunized splenocytes. Epitope mapping with splenocytes from BPN' Y217L immunized guinea pigs revealed no epitope regions with an average stimulation index of 3.0 in any of the animals tested (n = 5). This is consistent with our previous results for this enzyme, using splenocytes from animals immunized with 20 ug per dose of enzyme every two weeks for 10 weeks. Four out of five of the GP002 immunized animals responded with stimulation indices of greater than 3.0 to the modified epitope region in BPN' Y217L (peptide #25). The average response to this peptide was approximately 4.0. Peptide #25 is offset from the T cell epitope described in *B. lentus* by 2 amino acids. This is a result of creating linear 15-mers from the two sequences where there is a 2 amino acid contraction in *B. lentus* subtilisin as compared to the BPN' Y217L sequence. Primed splenocytes from guinea pigs also respond to peptide #24 that is 3 amino acids offset towards to N-terminus, and subsequently overlaps with the T cell epitope region. The creation of a T cell epitope in BPN' Y217L also resulted in

the display of a complex set of weak epitopes in the molecule. Peptides #25 and #75 induced average responses over the 5 animals that reached an S.I. of approximately 3.0. Five peptide regions induced average responses of approximately 2.5. Therefore, creation of a strong T cell epitope in this protein initiates an immune response that leads to the diversification of T cell responses to other regions of the molecule.

Antibody titers with BPN' Y217L and GP002

In order to test whether the modified enzyme could induce higher titers of antigen-specific IgG now that it carried a strong T cell epitope, guinea pigs were immunized with increasing doses of BPN' Y217L or GP002. Serum samples were taken every two weeks and titers were calculated. Average titers for four animals per group are shown in Figure 6. Immunization with the modified enzyme resulted in higher titers of antigen-specific IgG at early time points in the immunization protocol (Figure 6a). At later points during the immunization protocol, lower doses of the variant induced higher titers than the parent (Figure 6b, c, d). At four weeks, the 20 ug dose GP002 induced an average serum dilution titer of 5,000 versus no detectable IgG in the BPN' Y217L immunized animals. This difference between GP002 and BPN' Y217L has a p value of 0.03 when calculated using a two-tailed, non-parametric

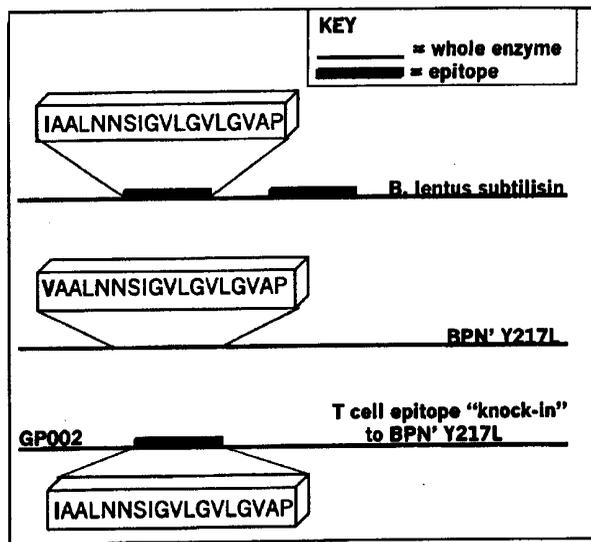


Figure 3
Schematic representation of the parent and modified enzyme variant. The first Guinea pig T cell epitope on *B. lentus* subtilisin has an analogous sequence (with a single amino acid change) in BPN' Y217L which is not detected in our assay as a T-cell epitope. A hybrid BPN' Y217L enzyme (GP002) was designed which contains the *B. lentus* subtilisin epitope.

Student's t-test. By ten weeks, the difference is no longer detected at the 5 and 20 µg doses but there is still a ten-fold difference in titers at the lowest dose.

Linear B Cell Epitopes BPN' Y217L v. GP002

A potential explanation for the increase in antibody titers is that the modification in GP002 results in a conformationally distinct molecule with unique surface regions, thereby increasing the total number of antibody binding sites. Guinea pig serum samples from animals immunized with 5 µg of enzyme were used to determine the position of linear B-cell epitopes in BPN' Y217L (Figure 7). The serum samples were drawn at 8 weeks, and whole protein titers are shown in Figure 6. Serum samples from four animals immunized with BPN' Y217L and four animals immunized with GP002 were tested on biotinylated 15-mer peptides (offset by 5 amino acids). Serum was diluted 1:1000 for all tests. Guinea pig IgG was detected with HRP-conjugated anti-IgG. The average optical densities for each peptide were divided by the background. The average result for each peptide is shown. The linear B-cell epitope regions recognized by both polyclonal serum samples are similar. The amino acid modification in GP002 would be in the region of peptides #13 to #16. If the amino acid modification produced a B cell epitope at this site, it does not cross-react onto the unmodified peptide regions tested here. The regions of the BPN' Y217L

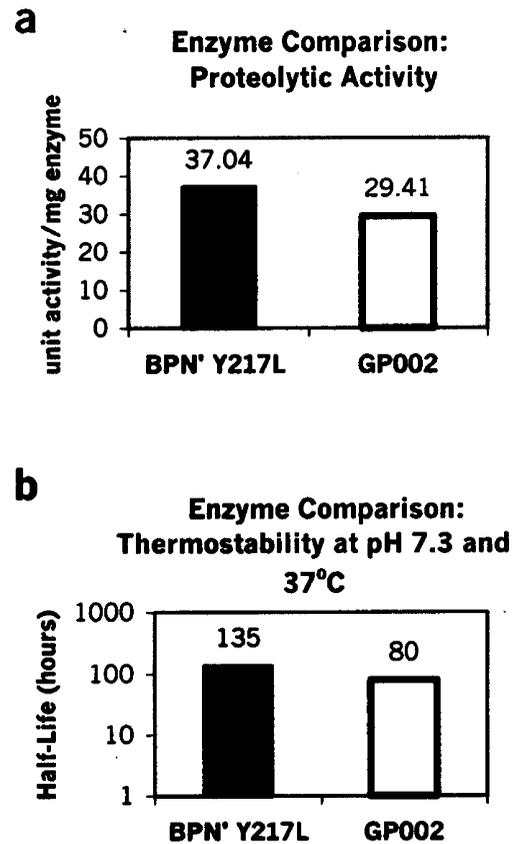


Figure 4
Proteolytic activity and stability of the variant GP002 as compared to the parent enzyme. The variant enzyme, GP002, was compared to BPN' Y217L for both proteolytic activity and thermostability. Figure 4A shows proteolytic activity as determined by a suc-AAFP-pNA hydrolysis assay. Figure 4B shows the results of the thermostability test performed at close to physiological conditions. Residual activity was determined over time, and the half-life was determined by linear regression.

molecule detected in this assay are predicted external loops (Dr. Richard Bott, Genencor International, personal communication). The major regions recognized, peptides #3 and 4, #17 and 18, and #46 and 47 are equivalently recognized by both serum sets. Of the 29 regions of the molecule showing an average two-fold increase in IgG binding over the background, 17 of these regions are detected more strongly by the GP002 sera. 5 peptide regions are uniquely detected by the BPN' Y217L serum, and 7 are uniquely recognized by the GP002 serum. There is a shift in one epitope region from peptides #38-43 where the GP002 antibody epitope runs from peptide 38 to peptide

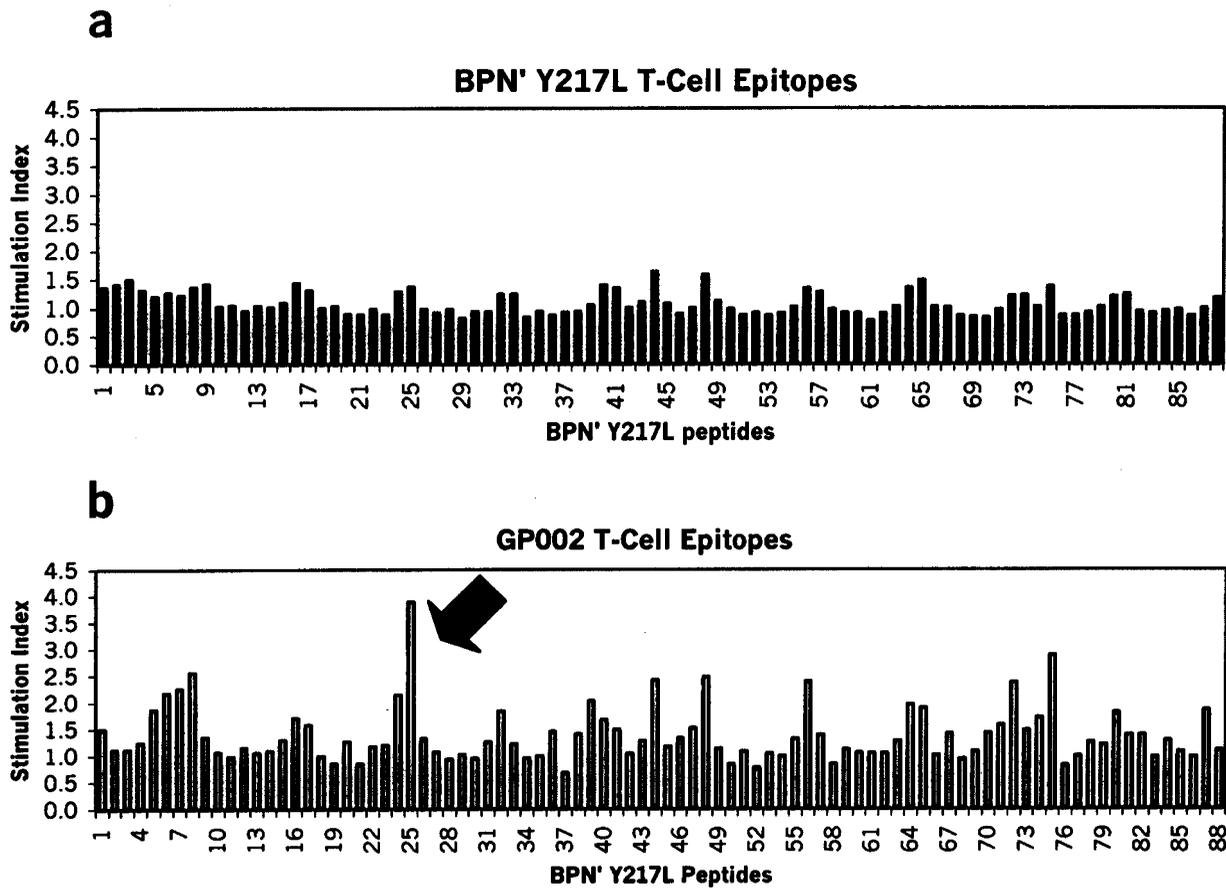


Figure 5

The introduction of a T cell epitope into the BPN' Y217L backbone results in a complex T cell epitope map. Two groups of five guinea pigs were immunized with 5 ug of either the parent or variant enzyme every two weeks for a total of three immunizations. At eight weeks, spleens were removed and splenocytes were tested in vitro for proliferative responses to BPN' Y217L peptides. The average proliferation is recorded above for each peptide across the five animals. Note that an epitope was created for the BPN' Y217L peptide number 25, where the single amino acid change was effected. The proliferative response was also boosted across the entire range of the enzyme.

41, and BPN' Y217L's runs from peptide 40 to peptide 43. This 10 amino acid shift forward indicates a slight difference in protein folding. This difference has little apparent effect on the other antibody epitopes delineated by this assay. This assay demonstrates that GP002-specific IgG antibodies readily cross-react with BPN' Y217L linear antibody epitopes.

BPN' Y217L and GP002 Priming Immunizations

It was of interest to determine whether increased titers of IgG antibodies to BPN' Y217L could be induced by priming animals with the T cell epitope-containing variant. Four sets of four guinea pigs were immunized with 5 µg of enzyme over a six-week period in series. The animals were immunized three times, once every two weeks. This proto-

col was chosen to correspond with the titer data presented in Figure 6. Group A was immunized three times with BPN' Y217L, Group B was immunized first with the GP002, and then twice with BPN'Y217L. Group C was immunized the first two times with the GP002, and the third time with BPN' Y217L.

Finally, Group D was immunized all three times with GP002. Two weeks after the last immunization serum samples were taken and ELISAs were performed to compare titers. All ELISAs were performed using the parent enzyme, BPN' Y217L, as a plate coating reagent. Results clearly show once again that immunization with GP002 induces over 10-fold higher levels of antigen-specific IgG than the unmodified parent enzyme (Figure 8). The differ-

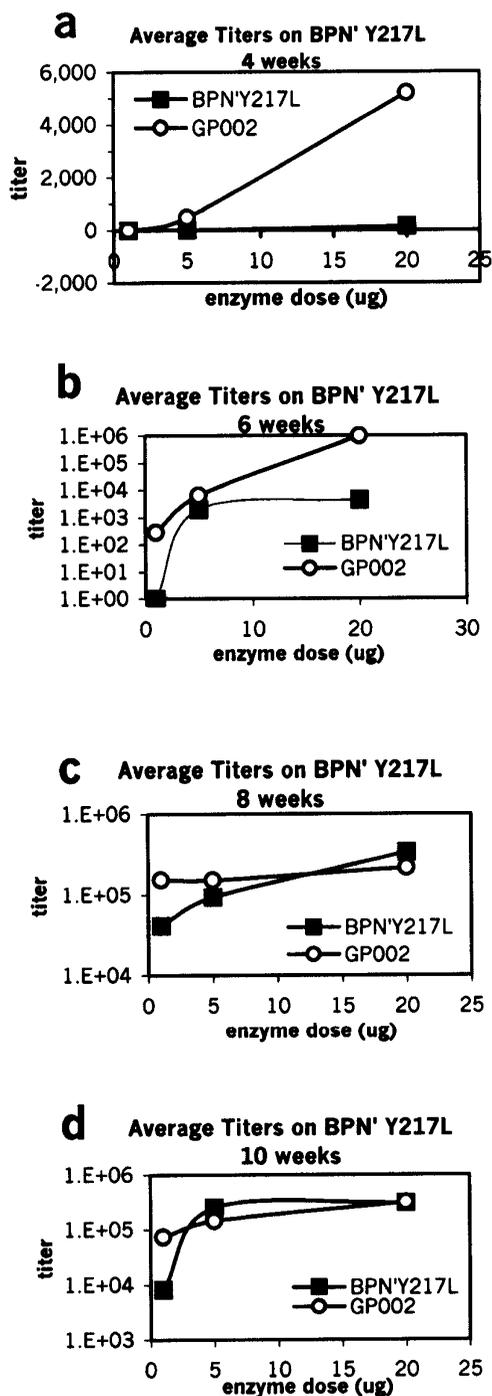


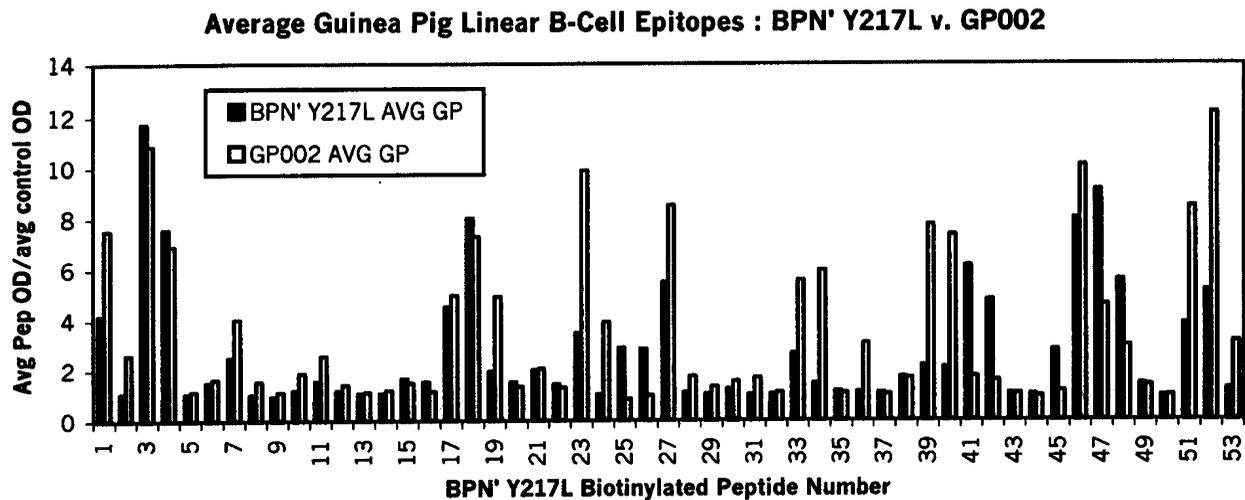
Figure 6
Antibody titers of BPN' Y217L v. GP002 immunized Guinea pigs. Guinea pigs were immunized with 1, 5, or 20 ug of BPN' Y217L or GP002. Serum samples were taken every two weeks over a ten-week time period. Dose responses are shown for the 4, 6, 8 and 10 week samples. Average titers for four animals per dose are shown.

ence between group A and D was highly significant ($p < 0.01$). In addition, a single immunization with the T cell epitope modified variant GP002 is sufficient to induce higher levels of parent-enzyme-specific IgG after subsequent immunizations with the unmodified parent enzyme. While the average titer increased by approximately two-fold, the difference between group A and B was not statistically significant. Each group contained only four animals, with appreciable scatter in the individual responses. Two immunizations with GP002 prior to immunizing with the parent enzyme did not improve the titers as compared to a single priming immunization with GP002. This result suggests that priming with the variant enzyme can induce higher levels of cross-reactive IgG antibodies than immunization with the unmodified variant alone. This experiment also confirms that GP002 immunized animals produce high titers of IgG that cross-react on the whole BPN' Y217L molecule.

Discussion

We have shown that a single conservative amino acid change in a bacterial subtilisin enzyme can create a strong T cell epitope in our guinea pig model. Immunization of guinea pigs with this modified variant results in an improved cross-reactive immune response to the unmodified parent enzyme in both antigen-specific IgG antibody production and T cell proliferative responses. The improved immunogenicity of the variant was not related to changes in the proteolytic capacity of the enzyme, because the variant exhibited 80% of the activity of the parent enzyme. The introduction of a single T cell epitope apparently allowed for the diversification of T cell responses to the protein because multiple epitopes were found in the BPN' Y217L sequence after immunization of animals with the variant (Figure 5). An increase in the complexity of the T cell response is consistent with an increase in overall antibody titer to the enzyme, as more T cell helper activity is potentially available. The antibody responses to the variant and parent enzymes were shown to be largely overlapping, indicating that the increase in the antibody titers seen were not due to the addition of a novel structure capable of being recognized by unique antibody specificities. This is shown by the last experiment where priming with the variant enzyme increased antibody titers to the parent enzyme upon subsequent immunizations.

It was of interest that the unmodified protease induces antigen-specific IgG in the absence of detectable immunodominant T cell epitopes. BPN' Y217L may possess weak T cell epitopes that are below our limit of detection in our cell culture assay. Our criterion for an epitope was an average stimulation index of 3.0. In addition, it is notable that both of the immunogens tested display considerable proteolytic activity. The proteolytic activity of the parent enzyme could be responsible for inducing T cell inde-

**Figure 7**

Linear B cell epitopes recognised by sera from guinea pigs immunized with the parent and the variant enzyme. Serum samples from animals immunized with the parent and the variant were used to detect IgG binding to peptides describing the sequence of BPN' Y217L. All sea was diluted 1:1000. Binding was detected with anti-guinea pig IgG. Absorbance results are averaged for four animals in each group.

pendent B cell differentiation via a number of different mechanisms [33–35]. Finally, we have seen that both chemically inactivated and an inactive variant of the parent protease used in these experiments are incapable of inducing IgG1 in a mouse model (data not shown). The proteolytic activity of these molecules also acts as an adjuvant for specific IgG production [36].

The ability to induce improved immune responses by the subtle changes described here could have a number of potentially vital applications. The use of a T cell epitope-modified whole protein immunogen confers a number of advantages. The first advantage is the priming of a diverse immune response to the protein that allows the generation of both a complex and robust T helper responses, and secondly the concomitant affinity maturation of the antibody response to naturally occurring antibody epitopes. This is especially relevant to the design of subunit vaccines for malaria and schistosomiasis where high titers of high affinity antibodies are usually the best defence against invading organisms [11,16,24]. Creating T cell epitopes in expressed proteins has been demonstrated here for Hartley strain guinea pigs, and for various inbred strains of mice [37,38]. Interestingly, the introduction of T cell epitopes from a hen-egg lysozyme or ovalbumin into mouse TNF-alpha also resulted in an increased cross-reactive antibody response in mice [38]. However, the protein construct was no longer cytotoxic for L929 cells, suggesting that the modified protein was not in the correct ter-

ary configuration. Incorrect tertiary configuration could lead to the production of specific antibodies that do not cross-react with the native molecule. T cell responses to the variants were not well characterized. In this paper, and in the report from Tsujihata [37] T cell epitopes were created within the molecule of interest, without affecting the activity of the enzyme. We created a naturally occurring T cell epitope by comparison to a related protein. In addition, we found that the creation of a T cell epitope in BPN' Y217L resulted in a complex pattern of T cell response to the enzyme. The challenge of utilizing this technique for the creation of subunit vaccines for the human population will be to create T cell epitopes within a protein of interest that will not have deleterious effects on the activity or the stability of the protein of interest, and that would be effective across a number of HLA-DR haplotypes [39].

The improved antibody response measured in this paper was increased titers of guinea pig polyclonal IgG. We did not measure potential differences in guinea pig IgG isotypes. Changing the interaction of peptide, MHC class II molecules, T cell receptors and other antigen-presenting cell specific ligand interactions to foster stronger, higher affinity T cell signalling has been shown in mice to skew T cell differentiation from Th2 to Th1-like patterns [40–50]. Differences in Th-differentiation patterns have downstream effects on the type of immune response mounted, and in particular on the isotypes of antibody expressed. This has been well documented for mice and humans, but

Average Log₂ Priming Titers for 5ug doses on BPN' Y217L

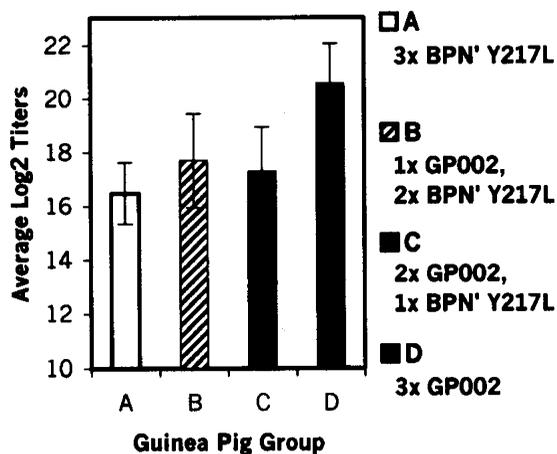


Figure 8

Priming of IgG responses to the parent enzyme, BPN' Y217L, by the T cell epitope modified variant GP002. Four sets of four Guinea pigs were immunized with 5 ug of enzyme on various schedules. All groups were immunized three times, once every two weeks. Group A was immunized all three times with BPN' Y217L. Group B was immunized first with GP002, and then twice with BPN' Y217L. Group C was immunized the first two times with GP002, and the third time with BPN' Y217L. Finally, Group D was immunized all three times with GP002. After testing the sera for whole IgG by ELISA on BPN' Y217L coated plates, titers were calculated

less is known about Th differentiation in Hartley strain guinea pigs.

In our system, we cannot distinguish if the amino acid change we have created has its effects on binding to the guinea pig MHC, interaction with the T cell receptor, or more efficient processing of the epitope from the protein precursor [51]. Any or all of these mechanisms could contribute to the enrichment for peptide-specific proliferative responses seen in vitro.

The ability to up regulate antibody and T cell responses to whole protein immunogens by the modification of T cell epitopes suggests that the converse would also be possible. Down regulation of T cell responses to functionally intact enzymes would have obvious implications for both existing and potential protein therapeutics.

Conclusions

The immunogenicity of an enzyme can be increased, while maintaining the majority of its stability and proteolytic activity, by incorporating a single amino acid change. The variant enzyme was found to have a major T cell epitope where none existed in the parent. In addition to the increased cellular response, humoral responses were increased at low doses, and as well for high doses early in the immunization schedule. Enhancing the T cell response to an enzyme, in addition to the humoral response, has applications in the construction of recombinant vaccines as well as in protein-based therapeutics.

Materials and Methods

Protease enzymes

GP002 was created by site-directed mutagenesis of the sequence of BPN' Y217L and expressed in *B. subtilis*. Enzymes were purified from fermentation broths utilizing the BioCAD 700E system (Perceptive Biosystems, Foster City, CA) using POROS HS/M resin (Perceptive Biosystems, Foster City, CA) on porous polystyrene beads, and was stored as stock solutions at 20 mg/ml in 20 mM MES, pH 5.5 with 1 mM CaCl₂ at -20°C.

Enzyme activity and stability measurements

Proteolytic activity was measured by hydrolysis of the substrate succinyl-AAPF-p-nitroanilide (suc-AAPF-pNA). Serial dilutions of the proteases were made in 100 mM Tris buffer, pH 8.6, containing 10 mM CaCl₂. Enzyme activity was measured at 25°C in 100 mM Tris/HCl, pH 8.6 containing 1.6 mM suc-AAPF-pNA (Bachem Bioscience, Torrance, CA), 0.0005% Tween-80 and 1% DMSO by monitoring the absorbance change at 410 nm using a spectrophotometer. Each dilution of enzyme was tested in triplicate. Three dilutions were tested for each enzyme. Activity was then determined by linear regression, and is reported as OD units/mg enzyme.

Comparative thermostability was determined for BPN' Y217L and GP002 by incubating the enzymes (0.5 ppm) at 37°C in 50 mM TES buffer, pH 7.3 containing 0.0005% Tween-80. Aliquots of the enzyme solutions were removed over time, diluted and assayed for activity using 1 mM suc-AAPF-pNA substrate in 100 mM Tris pH 8.6 containing 0.0005% Tween-80. The half-lives of the enzymes were then calculated using a linear regression.

Immunization protocols

1. BPN' Y217L, *B. lentus subtilis*, and GP002 immunizations

Guinea pig immunizations were outsourced to EL Labs in Santa Cruz, CA. Female Hartley outbred guinea pigs weighing 200–600 grams were used in all studies. There were three or four dosages of the appropriate enzyme and for each dosage there were four animals immunized. Each

group of four animals was immunized every two weeks, immediately after the removal of a serum sample. There were a total of six immunizations. The first immunization was in Complete Freund's Adjuvant. Incomplete Freund's Adjuvant was used for all subsequent immunizations. Each immunogen was provided as a stock solution at 20 mg/ml in 20 mM sodium acetate, 1 mM calcium chloride, and was diluted to 1, 5, 10, or 20 µg/ml in cold DPBS to a total volume of 1 ml. This was emulsified at a 1:1 ratio with CFA or IFA. Each guinea pig was immunized subcutaneously with a total of 500 µl of emulsion.

2. Priming study with BPN' Y217L and GP002

Guinea pig immunizations were out-sourced to EL Labs in Santa Cruz, CA. The following immunization protocol was followed: four groups of four Hartley guinea pigs were distinguished as groups A through D. All animals received 5 µg of immunogen. The first immunization was performed in CFA, then IFA for all subsequent immunizations. Each animal received a total of 500 µl of emulsion. All immunizations were performed subcutaneously using the following schedule: First immunization – Group A received BPN' Y217L, groups B-D GP002. Week two – Groups A and B received BPN' Y217L, Groups B and C received GP002. Week 4 – Groups A through C received BPN' Y217L, Group D received GP002. Serum samples were drawn at week 6.

3. T-cell epitope mapping for GP002 and BPN' Y217L

Guinea pig immunizations were outsourced to Strategic BioSolutions, Ramona, CA. Groups of 10 female Hartley strain guinea pigs were immunized every two weeks with 5 µg per dose of BPN' Y217L or GP002 in Complete Freund's adjuvant, then Incomplete Freund's as described above. Two weeks following the third immunization, animals were sacrificed and their spleens and serum samples collected.

ELISA protocols (whole protein IgG)

Medium binding EIA plates (Costar, Cambridge, MA) were coated with 10 µg/ml of acid-denatured enzyme in DPBS. Enzyme was coated onto the plates at 4°C overnight. Plates were then washed three times with PBS/0.25% Tween-20 (Sigma, St. Louis, MO). Plates were blocked with 1% BSA (Rockland, Gilbertsville, PA)/PBS at room temperature for 30 minutes. Ten-fold serial dilution of sera in 1% BSA/PBS were performed. The plates were covered and incubated at room temperature for two hours. Secondary biotin anti-IgG antibody (guinea pig IgG Fc specific, Jackson ImmunoResearch, West Grove, PA) was diluted 1:10,000 in 1%BSA/PBS. 100 µl of this dilution were placed per well on the plates. The plates were again covered and incubated at room temperature for 1 hour. Plates were washed (as above). Avidin-HRP (Jackson ImmunoResearch) was diluted 1:1000 in 1%BSA/PBS

and 100 µl per well were added to the plates. Plates were covered and incubated at room temperature for 30 minutes. The plates were washed (as above). The ELISAs were developed with ABTS in citrate buffer with 0.03% H₂O₂, at room temperature for 40 minutes and then read at OD = 405 nm. Titers were found by using a log-based slope equation where the O.D. = 0.5.

Peptides

1. Proliferation assays: 15-mer peptides offset by 3 amino acids describing the proteins of interest were obtained as a Pep Set from Mimotopes (San Diego, CA). Pep Sets were constructed for *Bacillus lentus* subtilisin (*B. lentus* subtilisin) and *Bacillus amyloliquifaciens* subtilisin (subtilisin BPN' Y217L). Peptides were resuspended in DMSO at 1 mM.

2. Linear epitopes: Biotinylated 15-mer peptides from obtained from Mimotopes (San Diego, CA). The 15-mer peptides were offset by 5 amino acids, and described the sequence of BPN' Y217L. The biotin moiety at the N-terminus was separated from the peptide sequence of interest by a 4 amino acid spacer. Biotinylated peptides were resuspended at 1 mM in DMSO.

T-cell epitope mapping of enzymes *B. lentus* subtilisin, BPN' Y217L and GP002

T cell epitope mapping was performed using splenocytes from immunized Hartley strain guinea pigs. Splenocytes from immunized guinea pigs were resuspended in AIM-V (Life Technologies Gaithersburg, MD) containing 100 units/ml penicillin, 100 µg/ml streptomycin, 1 mM glutamine, and either 10% autologous serum or 10% FCS (Sigma, St. Louis, MO). Using round-bottom 96 well plates (Costar, Cambridge, MA), either 5×10^5 or 1×10^6 cells/well in 200 µL of media. 5 µM of peptide were placed in each well. Each peptide was tested minimally in duplicate. DMSO was present at 0.5%. Control cultures contained 0.5% DMSO in the absence of peptide. The cultures were incubated at 37°C for 5 days. On day 5, cells were pulsed with tritiated thymidine at a concentration of 0.5 µCi/well. On day 6, cells were harvested and counted. A proliferative response was counted as positive if the average counts for a particular peptide were three times higher than the control levels.

Linear B-cell epitope mapping of enzymes BPN' Y217L and GP002

Biotinylated peptides were resuspended in 1 ml of DMSO (1 mM). A stock plate was made by diluting 10 µl of each peptide into 200 µl of PBS/0.025% Tween-20 (Sigma, St. Louis, MO). Streptavidin-coated plates (Pierce, Rockford, IL) were blocked with 1%BSA/PBS for 30 minutes at room temperature. Approximately 5 µM of biotinylated peptide was coated onto each well. Each peptide was tested mini-

mally in duplicate. Plates were incubated for one hour at room temperature and then washed three times. Guinea pig serum was diluted 1:1000 in PBS/Tween-20. 100 µl of dilute sera was placed into each well and plates were incubated at room temperature for one hour and then washed three times. The secondary antibody, anti-guinea pig IgG-HRPO (Jackson ImmunoResearch, West Grove, PA) was diluted 1:1000 in 1%BSA/PBS and 100 µl of dilute conjugate was added to each well. Plates were incubated at room temperature for 1 hour and then washed three times in PBS/Tween-20. Plates were then washed twice in PBS only. ELISAs were developed using ABTS in citrate buffer, 0.03% H₂O₂. Plates were then incubated at room temperature for up to 45 minutes and then read at 405 nm. OD readings for each peptide were compared to background readings with no peptide. Any peptide giving readings at least twice the background was considered a positive.

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