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Production and Purification of a Recombinant Human hsp60 Epitope Using the Cellulose-Binding Domain in *Escherichia coli*

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The heat shock protein hsp60 plays a functional role in insulin-dependent diabetes mellitus. The hsp60 epitope p277 (aa 437–aa 460) is effective in vaccinating mice against diabetes. A synthetic peptide gene (*p277*) that encodes the human hsp60 epitope was cloned to the 3′ end of the cellulose-binding domain gene (*cbd*). CBD-p277 was overexpressed in *Escherichia coli* and purified on a cellulose column. A methionine at the C-terminal end of CBD enabled CNBr cleavage between CBD and p277. After CNBr cleavage, free CBD and residual uncleaved CBD-p277 were recovered by cellulose chromatography. The p277 peptide was further purified on a RPC–FPLC column. The molecular weight of the recombinant peptide was confirmed by electrospray mass spectrometry. The recombinant peptide was found to be biologically active in assays involving clone C9 T-cell proliferation, lymph-node cell proliferation, and antibody production. Thus the use of CBD as an affinity tag and the utilization of affordable cellulose matrices offers an attractive method for the production and purification of recombinant peptides. © 1998 Academic Press

Peptides, which play an important role in many systems in nature, are produced commercially by the pharmaceutical industry. However, their chemical peptide synthesis is limited by high cost and low yield, particularly in the case of large peptides. Recombinant proteins have been successfully expressed in *Escherichia coli*, but small polypeptides (<10 kDa in size) are usually unstable in *E. coli* and rapidly degraded. Small peptides are therefore usually produced as fusion proteins (1) or in tandem repeats (2). Following production, the fusion protein is purified by a series of chromatographic steps and the peptide is cleaved from its carrier for recovery. The carrier protein may provide two functions: it stabilizes the expressed peptide and it acts as an affinity tag enabling easy purification of the fusion protein and/or the unwanted cleaved tag. For example, insulin has been produced by fusion to an IgG-binding protein tag, purification by affinity chromatography on an immobilized IgG column, and specific cleavage by trypsin (3). Moricin, an antibacterial peptide from the silkworm, *Bombyx mori*, was efficiently produced in *E. coli* as a fusion protein and released by chemical cleavage with CNBr (4). Many similar systems have been published in recent years (see 5 for review). However to date, affinity tags such as six histidine residues (6), glutathione *S*-transferase (7), or mannose-binding protein (8) have all required relatively expensive affinity matrices, limiting their commercial application in an industrial setting.

Shoseyov and Doi (9) isolated the cellulose-binding protein CBPA from the cellulolytic bacterium *Clostridium cellulovorans*. This major subunit of the cellulase complex binds to cellulose, but exhibits no hydrolytic activity. The *cbp*A gene was cloned and sequenced (10). Using PCR primers flanking the cellulose-binding domain (*cbd*), the latter was successfully cloned into an overexpression vector enabling us to overproduce the 17-kDa CBD in *E. coli*. The recombinant CBD exhibited strong affinity to cellulose (11). CBDs are also found in nature as discrete domains in cellulases (12,13). They provide a specific means of linking enzymes or other proteins to cellulose, a versatile and inexpensive immobilization matrix (14).

The spontaneous autoimmune process resulting in diabetes in the NOD mouse is first detectable as mild insulitis from about 1 month of age. In most female mice, the insulitis progresses to a penetrating intraislet infiltrate that leads to β-cell damage and overt

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insulin-dependent diabetes mellitus (IDDM), at about 4–5 months of age (15). Autoimmunity to hsp60 has been shown to play a functional role in diabetes: NOD mice spontaneously develop T cells responsive to the hsp60 peptide p277 (aa 437–aa 460) (16), and these T cells can adaptively transfer diabetes or, when attenuated, can be used to vaccinate mice against diabetes (17). Substituting valine for cysteine at positions 6 and 11 greatly enhances the stability of the peptide without affecting its immunological activity (Elias *et al.*, manuscript in preparation).

In this paper we report the production, purification, and biological activity of a recombinant human hsp60 epitope (p277) using the CBD as an affinity tag in *E. coli*.

MATERIALS AND METHODS

Bacterial strains and plasmids. *E. coli* XL1-Blue was used for cloning and *E. coli* BL21(DE3) (Novagen Inc., Madison, WI) was used for expression. The expression vector was pET3d (18, Novagen Inc.).

Enzymes and chemicals. Chemicals were purchased from Sigma Chemical Company (St. Louis, MO) unless otherwise stated. Enzymes were purchased from New England Biolabs Inc. *Taq* polymerase was purchased from Promega Inc.

TB medium was 1.2% Bacto tryptone, 2.4% Bacto yeast extract, 0.4% (v/v) glycerol, 0.017 M KH_2PO_4, and 0.072 M K_2HPO_4. M9 minimal medium was 0.6% Na_2HPO_4, 0.3% KH_2PO_4, 0.25% NaCl, 0.5% NH_4Cl, 20% glucose, 2 mM $MgSO_4$, 0.1 mM $CaCl_2$, and 1 mM thiamine–HCl.

Construction of pET-RI-CBD. Standard DNA manipulations were conducted according to Sambrook *et al.* (19). The *cbd* coding sequence was PCR amplified using *cbpA* as a template (11). The PCR products were analyzed on a 1% agarose gel and extracted from the gel using JET-Sorb extraction kit (Genomed Inc.). The primers were designed to eliminate one methionine at the N-terminal of CBD and resulted in a 6 amino acid deletion at the N-terminal of CBD, to abolish the *Nco*I site at the N-terminal of CBD and to create a *Kpn*I site at the C-terminal of CBD.

N-terminal primer was 5′-AAAAACATGTCAGTTGAATTCTACAA-3′; the primer contained an *Afl*III site (complementary to the *Nco*I site) and created an *Eco*RI site.

C-terminal primer was 5′-GGGGGGATCCTATGGTGCGGTACCAAG-3′; the primer created a *Kpn*I site and a stop codon followed by a *Bam*HI site. The 0.5-kb PCR product was digested with *Afl*III/*Bam*HI and cloned into pET3d which had been predigested with *Nco*I/*Bam*HI resulting in pET-RI-CBD. The ligation mixture was used to transform *E. coli* XL1-Blue. The sequence was confirmed and the plasmid was used to transform *E. coli* BL21(DE3) for expression of RI-CBD.

Construction of pETCBD-277. p277 (VLGGGVALLRVIPALDSLTPANED) is an hsp60 epitope in which two cysteins number 6 and 11 have been substituted with valines (20). A synthetic *p277* gene was constructed using four oligonucleotides designed to enable *Kpn*I/*Bam*HI cloning in pET-RI-CBD. In addition, a methionine within an *Nco*I site was included to enable CNBr cleavage of the fusion protein.

C-terminal sense strand was 5′-ATTCCAGCTTTGGATTCTTTAACTCCAGCTAATGAAGATTAAG-3′. C-terminal antisense strand was 5′-GATCCTTAATCTTCATTAGCTGGAGTTAAAGAATCCAAAGCT-3′. N-terminal sense strand was 5′-GATCGACCCCATGGGTACCATGGTTTTGGGTGGAGGTGTTGCTTTATTGAGAGTC-3′. N-terminal antisense strand was 5′-GGAATGACTCTCAATAAAGCAACACCTCCACCCAAAACCATGGTACCCATGGGGTC-3′.

The N-terminal and C-terminal oligonucleotides were mixed separately together (in separate tubes). The temperature was raised to 90°C and then gradually returned to RT. The two mixtures were phosphorylated and mixed together for ligation. The ligation mixture was digested with *Kpn*I/*Bam*HI and ligated into *Kpn*I/*Bam*HI-predigested pET-RI-CBD. This ligation mixture was used to transform *E. coli* XL1-Blue. The resultant plasmid, pETCBD-277, contained the *p277* gene as confirmed by sequencing.

Expression of RI-CBD and CBD-277 was essentially carried out using the pET system protocol for *E. coli* BL21(DE3). Proteins were analyzed by 12.5% SDS–PAGE according to Laemmli (21). Cells containing plasmids were grown overnight in M9 minimal medium containing 50 μg/ml ampicillin in an orbital shaker (250 rpm) at 37°C. The cells were diluted 1:50 in 500 ml TB medium containing 100 μg/ml ampicillin and were grown to an OD_{600} of 1.7, after which isopropyl-β-D-thiogalactopyranoside (IPTG) was added to a final concentration of 0.5 mM. After 4 h the cells (12 g wet wt) were harvested by centrifugation (5 min at 3000*g*) and resuspended in 10 ml of 50 mM Tris–HCl, pH 8, containing 10 mM EDTA. The cells were lysed in a French press, and inclusion bodies were collected by centrifugation at 10,000*g* for 15 min. Inclusion bodies (0.7 g protein) were washed with 10 ml and once in 20 mM Tris–HCl, pH 8, containing 1% (v/v) Triton X-100 and then washed three times with 20 mM Tris–HCl, pH 8, without detergent.

RI-CBD and CBD-277 were refolded as follows: inclusion bodies were dissolved in 20 mM Tris, pH 11.5 (7 mg/ml), and the solution was slowly titrated at RT (over a period of 2 h) to pH 7. The solution was centrifuged at 10,000*g* for 15 min to remove insoluble materials and then lyophilized. Binding of the refolded pro-

teins was tested as follows: 100 μg refolded protein was mixed with 1 mg cellulose in 1 ml 20 mM Tris buffer, pH 7, for 1 h at RT, after which the cellulose pellet was washed once with 1 ml of 1 M NaCl in the same buffer and twice with buffer only. The pellet was then mixed with 100 μl of Laemmli sample application buffer and boiled for 5 min and the supernatant was analyzed by SDS–PAGE.

CBD-277 was resuspended (10 mg/ml) in 70% (v/v) formic acid and a 100-fold molar excess of CNBr over 1 mol of methionine was added. The tube was sealed tightly and incubated 18 h in the dark at RT with slow agitation. The mixture was diluted 10 times with H_2O, frozen at −70°C, and lyophilized. The pellet was resuspended in 20 mM Tris, pH 7, at a concentration of 100 μg/ml and 1 g of cellulose (Sigmacell-20, Sigma) was added to every 10 mg of CBD and incubated using a rotator at RT for 1 h, allowing the free CBD and residual uncleaved CBD-277 to bind to the cellulose. The cellulose was centrifuged at 1600*g* and the supernatant containing p277 was collected.

Purification and ES-MS analysis of p277. The recombinant p277 was further purified by FPLC on a reverse-phase chromatography (RPC) column (Pharmacia LKB Inc., Uppsala, Sweden). A linear gradient was created using 0.1% trifluoroacetic acid (TFA) as buffer A and 100% acetonitrile as buffer B. p277 appeared as the major peak and was collected, dialyzed against 0.1 M HCl, frozen at −70°C, and lyophilized. Electrospray mass spectrometry (ES-MS) was performed using a VG platform mass spectrometer. Recombinant peptide (20 μg) was lyophilized and then dissolved in 100 μl water containing 0.05% (v/v) TFA. The resulting solution was analyzed by direct injection after calibrating the instrument with myoglobin.

Endotoxin removal. The lyophilized peptide was resuspended in H_2O and filtered through microcentrifuge filter NMWL-10 kDa (Sigma). The filtered peptide was lyophilized in pyrogen-free tubes, and 20 μg of the lyophilized peptide was assayed for endotoxin with a limulus amebocyte lysate (LAL) test kit (Kinetic-QCL, Bio Whittaker).

Mice. Inbred NOD/Lt mice were raised and maintained under specific pathogen-free conditions at the animal breeding center of the Weizmann Institute from a breeding nucleus originally provided by Dr. E. Leiter (Jackson ImmunoResearch Labs. Inc., Bar Harbor, ME). The onset of clinical IDDM in females of this colony began at about 4 months of age and reached a cumulative incidence of 80% or greater by 8 months of age. Female mice were used in these studies.

T-cell lines. The hsp60-specific T-cell clone C9 was derived from nonimmunized NOD spleen cells as described previously (17). The line was maintained in culture by repeated stimulation in the presence of hsp60 antigen and irradiated (3000 rad) syngeneic splenocytes as antigen-presenting cells. This C9 clone recognized the p277 epitope of the hsp60 antigen.

T-cell proliferation. Groups of five 6-week-old female NOD mice were immunized via the hind footpads with 100 μg of the recombinant peptide p277 emulsified in oil [incomplete Freund's adjuvant (IFA); Difco, Detroit, MI] as previously described (22,23). After 10 days the draining popliteal lymph nodes of the mice were removed and the T-cell proliferative response to the T-cell mitogen Con A (1.25 μg/ml; Sigma), or to the recombinant peptide (10 μg/ml) was assayed in vitro (17). Dose–response curves were done using up to 50 μg/ml peptide: the optimum response was obtained with 10 μg/ml. T-cell responses were detected by the incorporation of [^{3}H]thymidine added to the wells in quadruplicate cultures for the last 18 h of a 3-day culture. The stimulation index was computed as the ratio of the mean cpm of antigen-containing wells to control wells cultured without antigens or Con A. The standard deviations (SD) from the mean cpm were always less than 10%. Background cpm, in the absence of antigens, was 800–1500 cpm in the clone proliferation assay and 4000–6000 in the lymph-node cell assay.

Antibody ELISA. NOD mouse sera were tested for antibody binding to the recombinant p277 peptide, 4 weeks after a single immunization with the peptide. Assay plates (Maxisorp, Nunc Roskilde, Denmark) were coated with 10 μg/ml of the peptide in phosphate-buffered saline (PBS) for 4 h. Nonspecific binding was blocked with 5% BSA in PBS, and the plates were incubated with the test sera diluted 1:50. Antibody binding to the recombinant p277 peptide was detected using alkaline–phosphatase-conjugated anti-mouse IgG+IgM (Jackson ImmunoResearch Laboratories). A significant amount of antibody was defined as that having an OD_{405} greater than 0.25, which was 3 SD over the mean ELISA reading obtained in sera from 10 normal BALB/c mice.

RESULTS

Expression and purification of pET-RI-CBD and pETCBD-277 in E. coli BL21(DE3). pETCBD-277 was cloned as described in the legend to Fig. 1. Overexpression was performed in *E. coli* BL21(DE3) cells harboring pETCBD-277 and pET-RI-CBD and the proteins were accumulated in inclusion bodies. Following refolding, both RI-CBD and CBD-277 bound to cellulose, as evidenced by SDS–PAGE analysis. As expected, a 17-kDa RI-CBD and 20-kDa CBD-277 appeared as relatively pure proteins possessing the ability to bind specifically to cellulose (Fig. 2). Recombinant CBD-277 was produced to a level of 1.2 g/liter of *E. coli* broth containing 24 g of wet cells.

Purification of recombinant p277. p277 was successfully cleaved from CBD using CNBr. The cleavage was

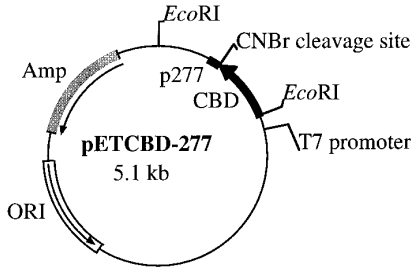

FIG. 1. Construction of pETCBD-277. The peptide gene was cloned at the 3′ end of the *cbd* gene. A CNBr cleavage site was inserted between CBD and p277.

almost complete, as evidenced by SDS–PAGE analysis of the reaction products (Fig. 3). The 17-kDa CBD protein appeared as the major band whereas only traces of the 20-kDa CBD-277 protein could be detected.

RPC–FPLC was performed to further purify p277 (Fig. 4). Up to 20 mg protein could be loaded onto 1 ml of RPC without any significant loss of resolution (not shown). After one chromatographic step on RPC–FPLC, the recombinant p277 was subjected to further analyses. The concentration of the recombinant peptide was determined by amino acid analysis. Approximately 100 mg of recombinant p277 with a purity of over 97% was produced from 1 liter of *E. coli* fermentation broth (Table 1). The molecular mass of the recombinant peptide (2390 Da) was confirmed by ES-MS (Fig. 5). In addition to this major peptide mass, two minor components of 2412 and 2428 Da were present in some of the spectra. These were putatively identified as sodium and potassium salt adducts, respectively. ES-MS analysis of the peptides after dialysis against 0.1 M HCl resulted in the expected 2390-Da peak without the 2412-Da and the 2428-Da peaks (data not shown), confirming our hypothesis.

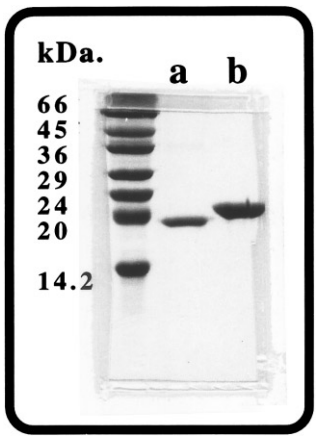

FIG. 2. Overexpression and purification of CBD-277. The 20-kDa fusion CBD-277 was purified from the inclusion bodies by cellulose affinity chromatography (b); (a) control CBD.

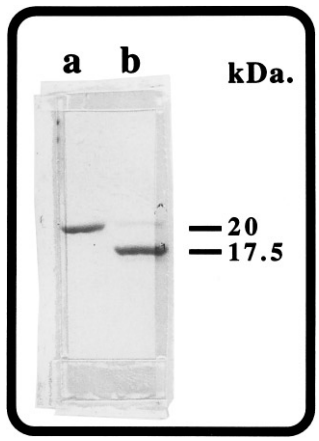

FIG. 3. CBD-277 cleavage by CNBr. Free CBD and residual uncleaved CBD-277 were recovered by cellulose-binding (b); (a) CBD-277.

Ultrafiltration of the recombinant peptide successfully removed the endotoxins: no gram-negative bacterial endotoxin was detected in the samples by LAL test.

T-cell clone proliferation. The proliferative response to the recombinant p277 peptide was tested using an established T-cell clone, C9, derived from prediabetic NOD mice splenocytes. This clone reacts specifically with the p277 epitope of the hsp60 antigen. The clone was tested in vitro against the recombinant p277 and against a preparation of CBD that did not contain any added antigen, as a control for bacteria-derived impurities that may affect the T cells. The peptide preparation stimulated the C9 clone, whereas the stimulating effect of the CBD preparation alone was similar to that of the background (BG, Fig. 6A).

Lymph-node cell proliferation. The direct immunogenicity of the recombinant peptide was further tested by immunizing NOD mice with the peptide and testing

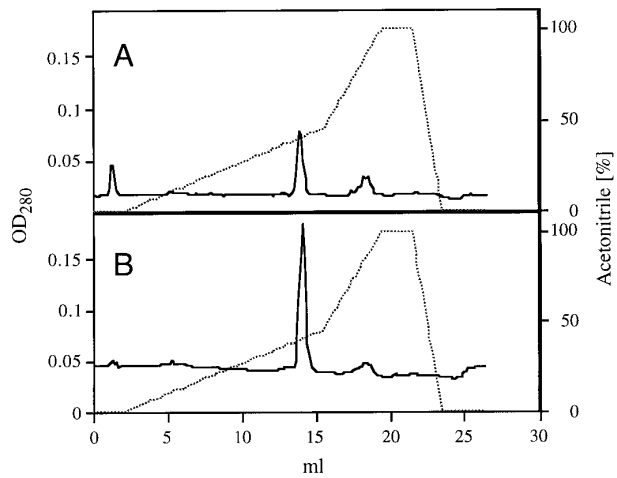

FIG. 4. Reverse-phase chromatography FPLC of the recombinant p277 before (A) and after (B) purification on RPC column.

TABLE 1

Production Table of p277 Expressed in *E. coli*

Purification step	Volume (ml)	Total protein (mg)	p277 (mg)
Fermentation broth	500	n.d.[a]	n.d.
Inclusion bodies	100	700	84
Refolding	100	600	72
CNBr cleavage and CBD removal	1200	120	60
RPC–FPLC	50	51	50

[a] Not determined.

the proliferative response of T cells from the draining lymph nodes (Fig. 6B).

Antibody production. Antibody response to the recombinant peptide was tested 4 weeks after immunization. Groups of eight mice were immunized with recombinant p277. The control mice were immunized with IFA alone or not immunized. After 4 weeks the mice were bled and their sera were tested in ELISA. Figure 6C shows the antibody-binding capacities of individual mice. All of the recombinant-p277-immunized mice developed specific antibodies against the peptide, whereas the IFA-immunized and nonimmunized mice did not.

DISCUSSION

A synthetic peptide gene (*p277*) encoding a human hsp60 epitope was successfully fused with the *cbd* gene. The resultant CBD-277 was overexpressed in *E. coli*, and a two-step purification procedure yielded highly purified recombinant peptide (Figs. 4 and 5). The authenticity of the peptide was confirmed by ES-MS, as well as by in vitro and in vivo assays. Approximately 100 mg of purified recombinant peptide per liter of culture was produced by this method (Table 1).

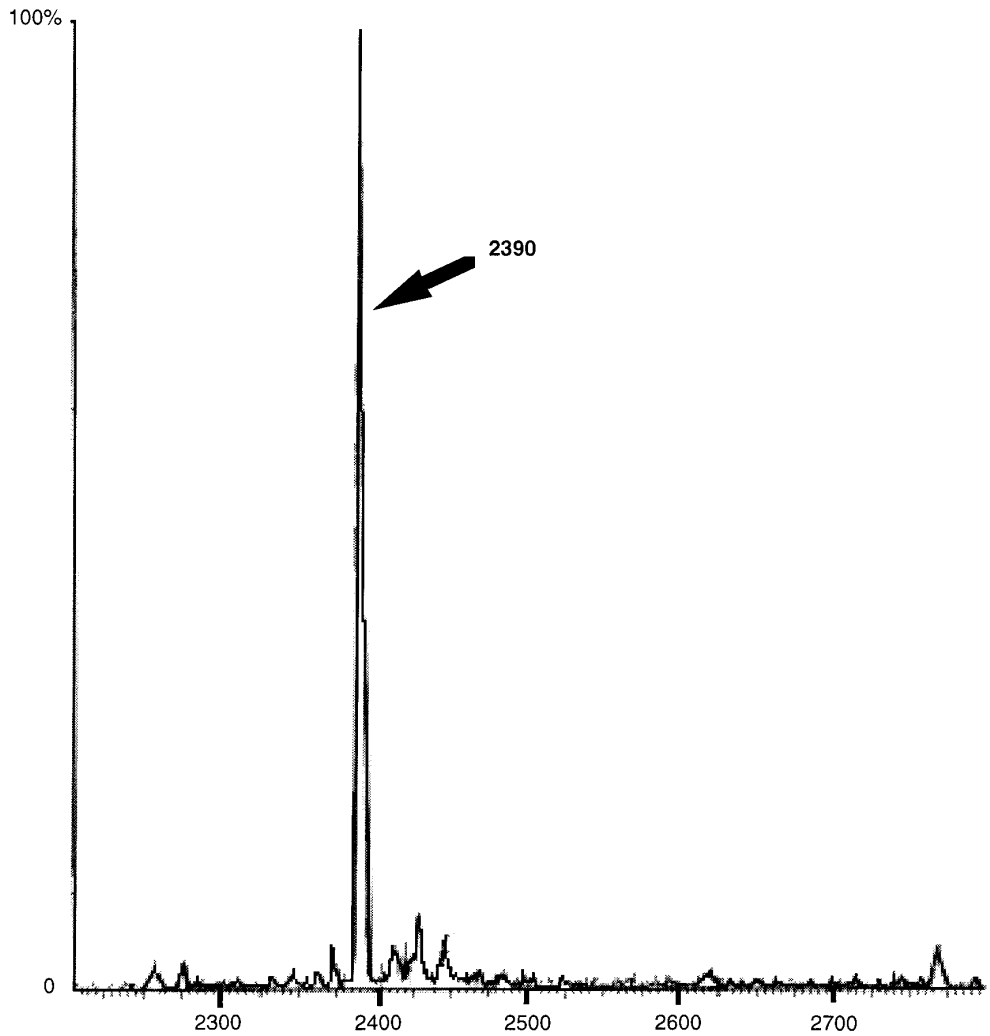

FIG. 5. Electrospray mass spectrometry of the recombinant p277. A major peak was found at the expected molecular weight of 2390 Da.

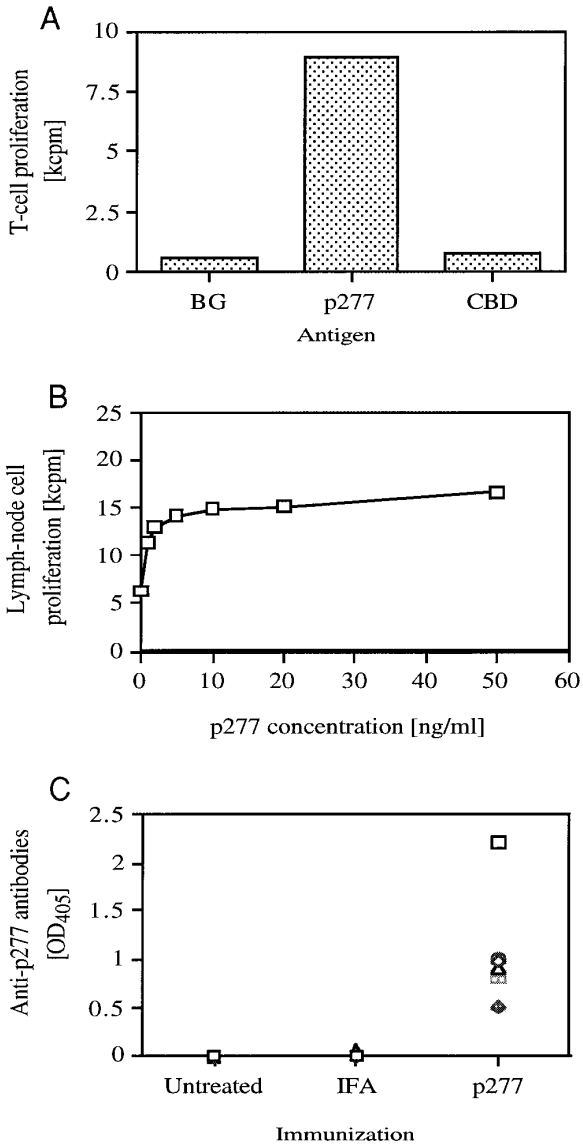

FIG. 6. Proliferation of T-cell clone C9 in response to recombinant p277 and CBD (BG, background) (A); lymph-node cell proliferation in response to increasing concentrations of recombinant p277 (B); ELISA of antibodies against recombinant p277 (C).

Recombinant peptide production methods have distinct advantages over solid-phase peptide synthesis, especially when peptides are over 20 amino acids in length. With the latter method, yields decrease with increasing length of the target peptide. During solid-phase peptide synthesis, a portion of the synthetic peptide remains incomplete, resulting in increased heterogeneity of the product with increasing peptide length. Hence, relatively high-molecular-weight synthetic peptides are difficult to purify by standard chromatographic methods. In contrast, the degree of purity and yield of peptides produced by recombinant peptide production methods are not affected by the length of the target peptide. Moreover, the final cost of peptides produced by synthetic methods increases with target length whereas in the recombinant methods, cost and length are unrelated.

The recombinant tandem-repeat method (2) generates homoserine or homoserine lactone residues at the C-terminus of the peptide (24), which may result in loss of peptide activity. In contrast, the CBD fusion method adds no residues to the target peptide.

In the CBD fusion expression system, the recombinant protein accumulates in inclusion bodies. Although some researchers have reported the advantages of soluble expressed protein (1), the accumulation of protein in inclusion bodies, as reported in this study, may offer some benefits: After a single washing of the inclusion bodies, nearly pure (over 95%) recombinant protein is recovered with no sign of degradation. Moreover, *E. coli* is well known to better tolerate toxic proteins/peptides when they are stored in inclusion bodies (25).

Cleaved CBD and uncleaved CBD-fused peptides are affinity removed from the reaction mixture by cellulose, which is very inexpensive and can be scaled up for large production protocols. This enables the final (and in fact, only) chromatographic step to be conducted under conditions in which the recombinant peptide is already a major component of the loading mixture on the reverse-phase column.

The major limitation of our method is that the peptide of interest must be devoid internal methionine residues if the peptide is to be released from the CBD by CNBr cleavage. This limitation can be overcome by utilizing different cleavage methods, such as factor Xa or enterokinase where cleavage sites can be easily engineered between the CBD and the target peptide. Another disadvantage of the recombinant method is the presence of bacterial endotoxins: these can be readily removed by 10-kDa cutoff ultrafiltration, as demonstrated in this study, provided that the peptide is smaller than 10 kDa.

In summary, the CBD and the availability of many inexpensive forms of cellulose matrices enable cost-effective production and purification of pure recombinant peptides, which may have applications in the pharmaceutical and the biotechnological industries.

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