# *Erwinia chrysanthemi* L-asparaginase: epitope mapping and production of antigenically modified enzymes

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This study shows that the antigenicity of *Erwinia chrysanthemi* L-asparaginase can be reduced by site-directed mutagenesis. Ten B-cell epitopes of the enzyme were identified using synthetic hexapeptides and polyclonal antisera from rabbits and mice. The region $^{282}$GIVPPDEELPG$^{292}$ near the C-terminus was an immunodominant epitope. Binding of two hexapeptides ($^{283}$IVPPDE$^{288}$ and $^{287}$DEELPG$^{292}$) to the antibodies was dependent on Pro$^{285}$, and Pro$^{286}$, since their replacement by almost any other amino acid resulted in reduced binding. The other residues were less important for binding the antibodies, as binding was relatively unaffected by amino acid substitutions. Three site-directed mutant enzymes, P285T (proline-285 → threonine etc.), P286Q and E288A, were expressed in *Escherichia coli*. The purified enzymes had subunit $M_r$ values of 35000. The pI values of P285T, P286Q and the wild-type enzymes were 8.6, and that for the mutant E288A was 9.2. The $k_{cat.}$ and $K_m$ values for the mutants P286Q and E288A with L-asparagine and L-glutamine were comparable with those of the wild-type enzyme. The $K_m$ values for the mutant P285T with both substrates was similar to that of the wild-type enzyme, whereas the $k_{cat.}$ was reduced by 2-fold with L-asparagine and by 4-fold with L-glutamine. The change proline → threonine reduced the antigenicity of the enzyme by 8-fold, as shown in sandwich e.l.i.s.a.s. using monoclonal antibodies raised against the wild-type enzyme.

## INTRODUCTION

Interest in L-asparaginase (EC 3.5.1.1) has grown considerably since this enzyme was found to have anti-tumour activity (Kidd, 1953; Broome, 1961, 1963). The bacterial L-asparaginases from *Erwinia chrysanthemi* and *Escherichia coli* are effective in treating acute lymphoblastic leukaemia and lymphosarcoma, but their use against other forms of leukaemia or solid tumours is limited, since remissions are invariably of short duration (Wade and Phillips, 1971). L-Asparaginase catalyses the hydrolysis of L-asparagine. Some tumour cells are deficient in L-asparagine synthetase and cannot synthesize sufficient L-asparagine. These cells are dependent on extracellular sources of the amino acid in order to complete protein synthesis (Keating et al., 1993). They can therefore be destroyed by starving them of L-asparagine by the administration of L-asparaginase.

Treatment with the enzyme is often accompanied by toxic side effects, such as liver dysfunction and pancreatitis (Eden et al., 1990). The enzymes from *Vibrio succinogenes* and guinea-pig serum lack hepatoxicity (Distasio et al., 1982) and do not possess L-glutaminase activity, suggesting that this activity in the *Er. chrysanthemi* and *E. coli* enzymes may be responsible for some of their toxic side effects. Another of the limiting factors of L-asparaginase treatment is the development of hypersensitivity, which ranges from mild allergic reactions to anaphylactic shock. The allergic reactions are normally treated by substitution with one of either the *Er. chrysanthemi* or the *E. coli* enzymes, as they show no immunological cross-reactivity (Hrushesky et al., 1976).

The study of antigenic determinants has widespread importance in vaccine production, medical diagnostics and basic biological research (Goodman, 1989; Konrad, 1989; Stern, 1991). Peptide-based assays for epitope mapping have been used extensively to identify antigenically important regions in proteins (Alexander et al., 1992), although no attempts to reduce the antigenicity of a therapeutic protein have been reported. A combination of synthetic peptide analysis and site-directed mutagenesis have been used to modify the antigenicity of myohaemerythin (Alexander et al., 1992). Residues that were identified as essential for antibody binding in peptide assays were also essential for antibody binding to the native protein.

One way of reducing the immunogenicity of a therapeutic protein is to covalently attach poly(ethylene glycol) derivatives. In the case of asparaginase this was found to reduce the immunogenicity in animal experiments (Abuchowski et al., 1984; Wada et al., 1990; Kodera et al., 1992). An alternative strategy to reduce the immune response would be to design a series of antigenically distinct, but catalytically similar, L-asparaginases. Treatment would involve the successive substitution of antigenically different forms of the enzyme to evade a strong immune response. This would decrease the allergic reactions associated with repeated use of the enzyme as well as expand the time over which the enzyme can be effectively administered. In this study the continuous epitopes of *Er. chrysanthemi* L-asparaginase were located by the binding of a set of overlapping synthetic peptides spanning the entire amino acid sequence to antibodies raised against the native enzyme. Residues within an immunodominant region that were essential for reduced binding of the peptides to the antibodies were identified using the replacement-net technique (Geysen et al., 1987, 1988). Those residues were further investigated by their replacement in the native protein. Three recombinant mutant enzymes P285T (proline-285 → threonine etc.), P286Q and E288A were constructed by site-directed mutagenesis and their catalytic and antigenic properties determined.

Abbreviation used: Fmoc, fluorenylmethyloxycarbonyl; P285T (etc.), proline-285 mutated to threonine.

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## MATERIALS AND METHODS

### Materials

*Er. chrysanthemi* L-asparaginase was obtained from this Institution. *E. coli* L-asparaginase was supplied by Sigma Chemical Co. The epitope-scanning kit was from Cambridge Research Biochemicals (Cambridge, U.K.). Polyclonal antibodies were raised against both *Er. chrysanthemi* L-asparaginase in rabbits (RpAb 1, RpAb 2) and mice (MpAb 1, MpAb 2) and against the *E. coli* enzyme in rabbit (RpAb 3). Monoclonal antibodies were raised against the *Er. chrysanthemi* enzyme. All antisera were used without purification unless otherwise stated. Horseradish peroxidase-linked antibody conjugates were from Amersham International, restriction enzymes were obtained from BRL/GIBCO (Paisley, Renfrewshire, Scotland, U.K.), Sequenase was from Cambridge Bioscience (Cambridge, U.K.). All other reagents were of the highest grade available commercially.

### Methods

#### Immunization and antibodies

Protein and peptide antibodies were raised by the method of Newell et al. (1988). Purified immunoglobulin was obtained by affinity chromatography with Protein G–Sepharose (Pharmacia).

#### Epitope mapping

Sequentially overlapping hexapeptides spanning the entire L-asparaginase sequence were synthesized on polyethylene rods by the addition of amino acids as fluorenylmethyloxycarbonyl (Fmoc)-protected pentafluorophenyl esters. The syntheses were carried out as described by Geysen et al. (1987). The L-asparaginase sequences $^{283}$IVPPDE$^{288}$ and $^{287}$DEELPG$^{292}$ were synthesized as a series of substitution peptides in a replacement net synthesis. This involved synthesizing peptide analogues of the parent peptide in which a different residue was iteratively replaced by the 19 alternative amino acids. Binding of the peptides to polyclonal antibodies was measured by e.l.i.s.a. (Geysen et al., 1987).

#### Synthesis of a free peptide and peptide conjugation to carrier protein

The peptide $^{282}$GIVPPDEELPG$^{292}$ (C) of the L-asparaginase sequence was synthesized manually by Fmoc solid-phase chemistry (Merrifield, 1963). The peptide was synthesized as a C-terminal amide using 4-(2′,4-dimethoxyphenyl-Fmoc-aminoethyl)-phenoxy-resin. Cleavage from the resin was with 95% (v/v) trifluoroacetic acid for 20 min at room temperature. Characterization of the peptide was by reverse-phase h.p.l.c., with 98% purity obtained. The peptide sequence was verified by fast-atom-bombardment m.s. (Wait et al., 1991). The terminal cysteine residue was used for coupling the peptide to the carrier protein thyroglobulin by using *m*-maleimidobenzoyl-*N*-hydroxysuccinimide (Green et al., 1982). Mouse polyclonal antibodies were raised using 10 $\mu$g of the peptide–carrier conjugate.

#### E.l.i.s.a.

Three types of e.l.i.s.a were used to test the ability of antibodies to interact with different forms of the antigen.

(i) Screening of the rod-bound peptides. Polyethylene-bound peptides were screened for binding to antibody as described in the manufacturer's instructions for the epitope mapping kit (Cambridge Research Biochemicals, Cambridge, U.K.).

(ii) Indirect e.l.i.s.a.: binding of antibody to antigen-coated plates. Protein antigen was coated on to microtitre plates (Nunc Immunolon-2), using 100 $\mu$l/well at a concentration of 5 $\mu$g/ml in coupling buffer (15 mM $Na_2CO_3$/35 mM $NaHCO_3$, pH 9.6), overnight at 4 °C. Alternatively, free peptide in PBS (8 mM $Na_2HPO_4$/1.5 mM $KH_2PO_4$/137 mM NaCl/2.7 mM KCl, pH 7.4) was coated overnight at 37 °C and then fixed with methanol at room temperature for 10 min. The plates were washed twice between all subsequent incubations with 300 $\mu$l of PBS, containing 0.1% (v/v) Tween-20 per well. Residual binding sites were blocked for 1 h with 100 $\mu$l of supercocktail buffer [1% (w/v) BSA/1% (w/v) ovalbumin/0.1% (v/v) Tween-20 in PBS]. Serially diluted test antibody in PBS was added, incubated for 1–1.5 h, and the plates washed. The plates were incubated for a further 1 h with 100 $\mu$l/well of horseradish peroxidase anti-specific $F(ab)_2$ conjugate and developed using 120 $\mu$l/well of freshly prepared 2,2′-azinobis-(3-ethylbenzthiazoline-6-sulphonic acid) substrate solution [0.5 mg/ml in 0.03% (v/v) $H_2O_2$/0.1 M $Na_2HPO_4$/0.08 M citric acid, pH 4.0]. The $A_{450}$ of the coloured product was determined using a Titertek Multiscan plate reader.

(iii) Sandwich e.l.i.s.a.: Binding of antibodies to ligand-captured antigen. Purified monoclonal antibody raised against the wild-type enzyme was coated on to microtitre plates at 5 $\mu$g/ml in coupling buffer (100 $\mu$l/well) by incubation overnight at 4 °C. After the washing, and blocking steps the wells were incubated for 1–1.5 h with serially diluted test antigen (100 $\mu$l/well; 0–20 ng/ml). The bound antigen was detected with an optimized concentration of polyclonal antiserum (1:500) raised against the wild-type enzyme. The e.l.i.s.a. was then continued as described above for the indirect e.l.i.s.a.

#### Construction of the L-asparaginase expression vector and site-directed mutagenesis

The plasmid expression vector containing the L-asparaginase gene pASN400 was kindly provided by Mr. I. R. Wilkinson of this Division. The vector was derived by deleting the 2.3 kb fragment (*Bam*H1/*Nru*1) of pASN326 (Gilbert et al., 1986) which was then inserted into the *Bam*H1/*Nru*1 site in the linker region of pMTL22p (Chambers et al., 1988).

Site-directed mutagenesis was performed on L-asparaginase DNA cloned into M13mp8 (Sambrook et al., 1989). Oligodeoxyribonucleotides for use as sequencing primers and in site-directed mutagenesis were synthesized using an Applied Biosystems Model 380A DNA synthesizer. The three oligonucleotides for each of the mutations P285T, P286Q and E288A were:

5′-GG TAT TGT GAC GCC GGA TG-3′

5′-T TGT GCC GCA GGA TGA AGA-3′

5′-C GCC GGA TGC AGA GCT GC-3′

Site-directed mutagenesis to produce the single mutations was performed on single-stranded recombinant M13 template DNA by the primer-extension method as described by Carter et al. (1985). Double-stranded phage DNA containing the L-asparaginase gene was digested with *Sal*1/*Nru*1 and a 0.9 kb fragment, containing the mutation, was isolated by agarose-gel electrophoresis. This DNA fragment containing the mutation was used to replace a similar fragment from the wild-type gene by subcloning in the vector pASN410. The vector pASN410 was derived from pASN400, which was constructed by replacing the wild-type 0.9 kb *Sal*1/*Nru*1 fragment of L-asparaginase with

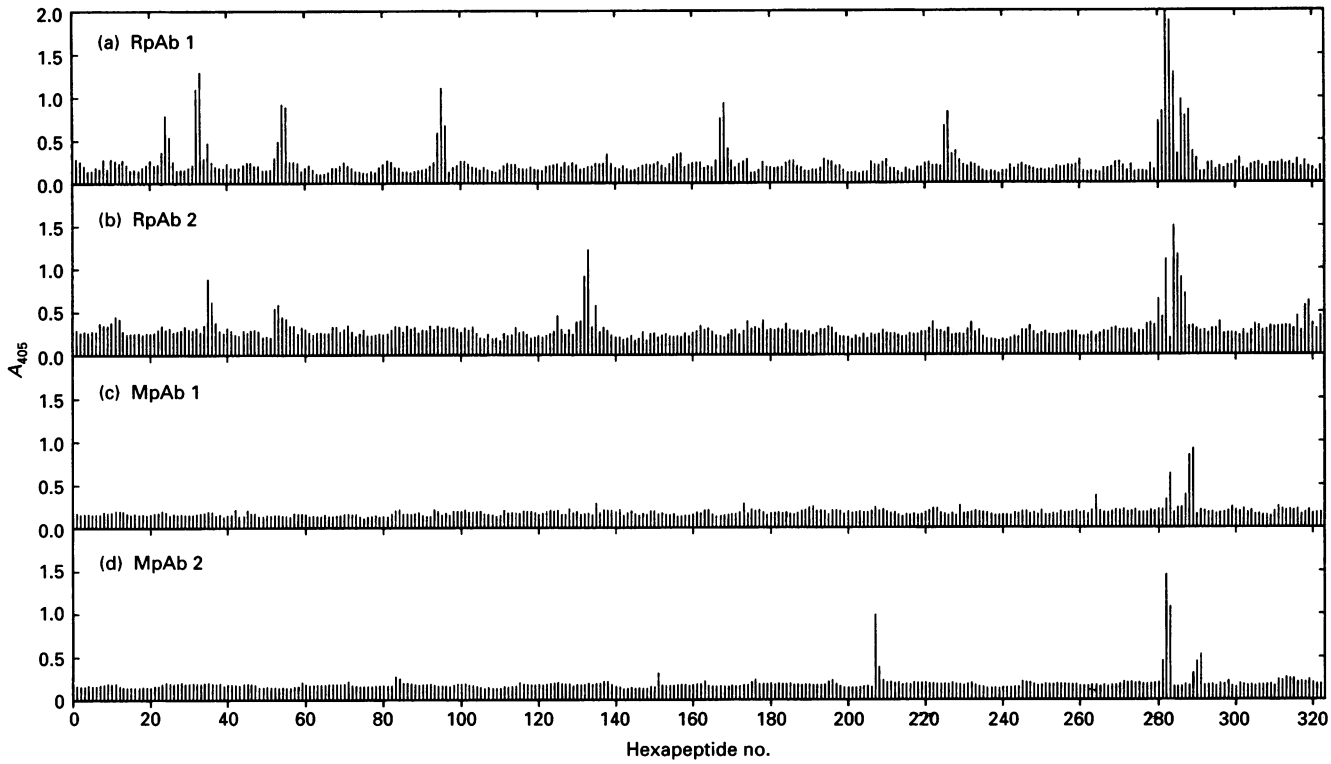


**Figure 1 Binding of overlapping hexapeptides to antiserum raised against *Er. chrysanthemi* L-asparaginase**

Peptides are identified by their number in the sequence commencing with the N-terminal residue of the native protein. (**a**) Reactivity of rabbit polyclonal antiserum (RpAb 1, diluted 1:500); (**b**) reactivity of purified rabbit polyclonal IgG (RpAb 2, 20 $\mu$g ml$^{-1}$); (**c**) reactivity of mouse polyclonal antiserum (MpAb 1, diluted 1:200); (**d**) reactivity of mouse polyclonal antiserum (MpAb 2, diluted 1:500).

linker oligonucleotides designed to ligate the *Sal*1/*Nru*1 sites. The presence of the mutations, and the absence of spurious mutations, in the 0.9 kb insert were confirmed by DNA sequencing using the dideoxy-chain-termination method of Sanger et al. (1977).

### Enzyme purification

Recombinant *Er. chrysanthemi* L-asparaginase expressed in *E. coli* was purified by a modification of the method of Goward et al. (1992). Protein from the cell extract in 40 mM sodium phosphate, pH 6.0, was purified by cation-exchange chromatography on a Mono-S HR 10/10 column (10 cm × 1 cm) (Pharmacia). Bound protein was eluted with 40 mM sodium phosphate, pH 6.8.

### Enzyme activity

L-Asparaginase activity was determined using a Technicon Autoanalyser as described by Wade and Phillips (1971), but incorporating the Berthelot colour reaction (Gordon et al., 1978). One unit of L-asparaginase was defined as 1 $\mu$mol of $NH_3$ produced/min at 37 °C. Protein concentrations were determined by the Folin method of Lowry et al. (1951), with BSA as standard. Column eluates were monitored by measuring the $A_{280}$.

Kinetic constants were determined in duplicate, and were calculated by non-linear regression analysis using the program ENZFITTER (Biosoft, Cambridge, U.K.).

### Gel electrophoresis

The purity of L-asparaginase was determined by SDS/PAGE using a PhastGel apparatus (Pharmacia) and 10–15% acrylamide PhastGels stained with Coomassie Brilliant Blue R250. The $M_r$ of L-asparaginase was compared with that of low-$M_r$ markers (Pharmacia). The pI values of the enzymes were determined using Servalyt–Precotes (Serva, Heidelberg, Germany), with a pH range of 3–10, according to the supplier's instructions. pI values were compared with markers in the pH range 3–9.5 (Bio-Rad).

## RESULTS AND DISCUSSION

### Epitope mapping

Ten continuous epitopes were identified when overlapping hexapeptides spanning the entire L-asparaginase sequence were allowed to bind polyclonal antibodies (Figure 1). These antibodies were raised against the native enzyme in two rabbits (RpAb 1 and RpAb 2) and two mice (MpAb 1 and MpAb 2). Some of the sites included multiple overlapping determinants, whereas residues 282–292 near the C-terminus of the protein represented an immunodominant epitope to which most of the immune response was directed. Residues $^{285}$PPDEE$^{289}$ were common to all overlapping epitopes in the region 282–292. The immunodominant region was unique to the *Er. chrysanthemi* enzyme, since rabbit polyclonal antibodies raised against *E. coli* L-asparaginase did not bind peptides in that region (Figure 2).

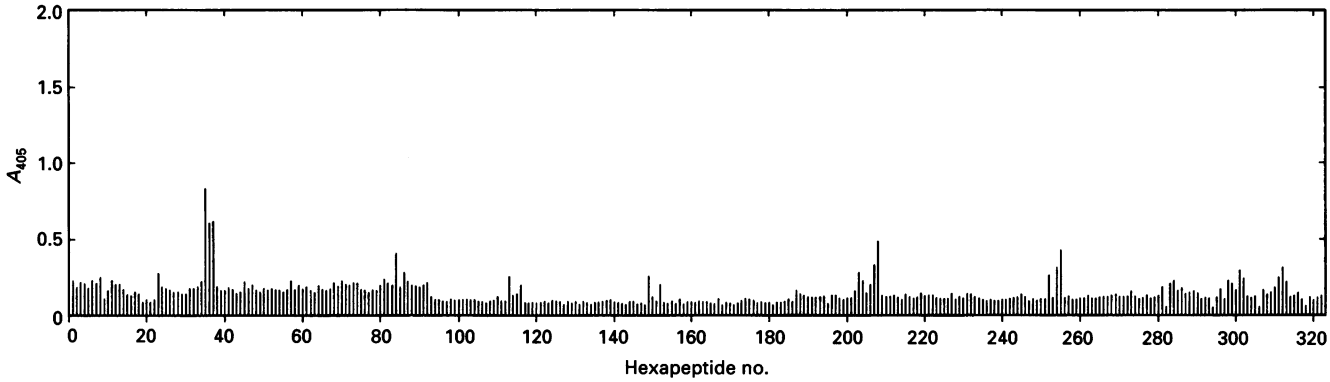


**Figure 2 Binding of the overlapping hexapeptides to antiserum raised against *E. coli* L-asparaginase**

The reactivity of rabbit polyclonal antiserum (RpAb 3, diluted 1:500) is shown. Peptides are identified by their number in the sequence commencing with the N-terminal residue of the native protein.

Four epitopes were identified with antibodies raised against the *E. coli* enzyme. They corresponded to $^{37}$DTLI$^{40}$, $^{84}$DDVDGV$^{89}$, $^{208}$VFDVRG$^{213}$ and $^{255}$VSVRGI$^{260}$. Two of these epitopes, residues 37–40 and 208–213 were also recognized by polyclonal antibodies raised against the *Er. chrysanthemi* enzyme. This cross-reactivity may be due to structural similarities between the two enzymes, since both have similar amino acid sequences, with 42% of the residues being identical (Maita and Matsuda, 1980; Minton et al., 1986). The cross-reacting epitopes may also contain regions of antigenic equivalence. Sequence alignments indicate that the epitope $^{37}$DTLI$^{40}$ of the *Er. chysanthemi* enzyme sequence corresponds to $^{33}$ENLV$^{36}$ in the *E. coli* enzyme. Similarly, for the epitope $^{205}$VFDVR$^{212}$ the corresponding sequence in the *E. coli* enzyme is $^{199}$PFDVS$^{203}$. Cross-reaction may also have occurred due to the multispecificity of the antibody-combining sites.

### Verification of the immunodominant region using a free peptide $^{282}$GIVPPDEELPG$^{292}$ in e.l.i.s.a.

A free peptide spanning the immunodominant epitope bound polyclonal antibodies raised against both native *Er. chrysanthemi* L-asparaginase and against the peptide (Figure 3). Binding of the free peptide to antiprotein antibodies provided further indication that the peptide may be an epitope of the protein. The antipeptide antibodies did not cross-react with *E. coli* L-asparaginase, indicating that the region was unique to the *Er. chrysanthemi* enzyme. Modification of this region would be expected to alter the antigenicity of the enzyme.

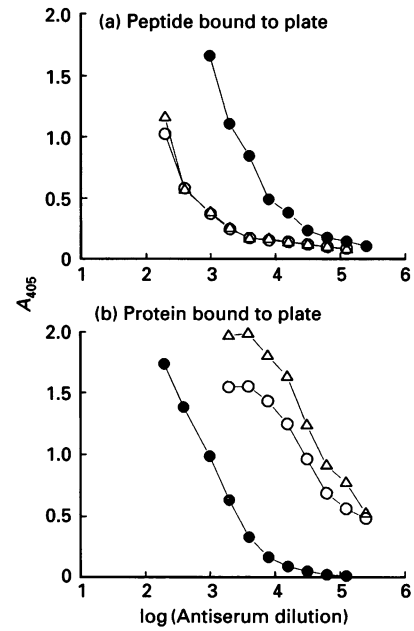


**Figure 3 E.l.i.s.a. with mouse polyclonal antiserum raised against peptide 282–292 and antisera raised against L-asparaginase (RpAb 1 and MpAb 2)**

(**a**) Binding with peptide $^{282}$GIVPPDEELPG$^{292}$ immobilized on microtitre plates; (**b**) binding with *Er. chrysanthemi* L-asparaginase immobilized on microtitre plates. ●, Mouse polyclonal antibodies raised against the peptide; ○, mouse polyclonal antibody MpAb2; △, rat polyclonal antibody RpAb1.

### Replacement-net analysis

The contribution of each amino acid residue within the immunodominant region for binding to the antibody was studied. All the residues in each of two hexapeptide sequences $^{283}$IVPPDE$^{288}$ and $^{287}$DEELPG$^{292}$ were sequentially replaced with the other 19 naturally occurring amino acids (Figures 4 and 5). Overall, those residues within peptide $^{283}$IVPPDE$^{288}$ that were essential for recognition by the rabbit and mouse polyclonal antibodies were Pro$^{285}$ and Pro$^{286}$ (Figure 4). Replacement of each of these residues by most of the other amino acids resulted in binding being reduced to less than 50% of that of the parent peptide. Similarly, Pro$^{291}$ in peptide $^{287}$DEELPG$^{292}$ was essential for binding to the antibodies, whereas some substitutions for each of the residues in the sequence $^{287}$DEEL$^{290}$ and for Gly$^{292}$ showed decreased binding to the antibodies (Figure 5). Those amino acid replacements that reduced binding of the peptide to the antibody were change of charge, or aromatic hydrophobic to aliphatic hydrophobic, or a small residue to a large residue.

### Site-directed mutagenesis

The mutations P285T, P286Q and E288A in the immunodominant region were selected, since the replacement residues were those present in corresponding positions in the *E. coli* L-asparaginase sequence (Bonthron et al., 1990). These mutations would be less likely to alter the overall structure or catalytic function of the enzyme. The residues Pro$^{285}$ and Pro$^{286}$ were

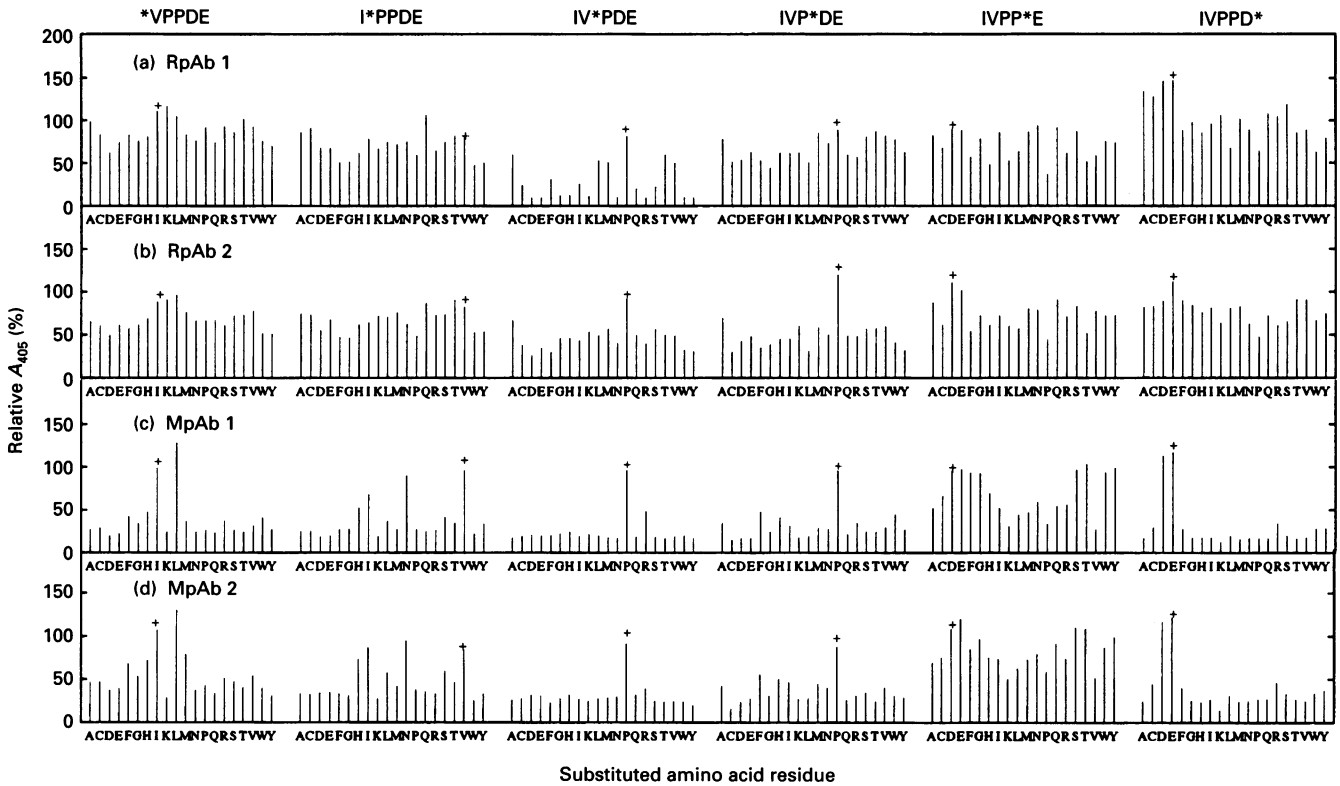


**Figure 4 Replacement-net analysis for the peptide $^{283}$IVPPDE$^{288}$**

Antigenic peptides of the sequence $^{283}$IVPPDE$^{288}$ with each of the amino acids replaced sequentially by one of the 20 amino acids from Ala to Tyr (the amino acid replacement is denoted by *). Results are of relative absorbance expressed as a percentage of that of the parent peptide (IVPPDE) versus each of the peptides containing substituted amino acids. Binding is with polyclonal antiserum raised against native *Er. chrysanthemi* L-asparaginase. E.l.i.s.a. values for the six copies of the parent sequence have been averaged and taken as 100%. '+' denotes the position of the parent peptide. (**a**) Binding with rabbit polyclonal antiserum (RpAb 1, diluted 1:200); (**b**) binding with purified rabbit polyclonal antibodies (RpAb 2, 20 $\mu g \cdot ml^{-1}$); (**c**) binding with mouse polyclonal antiserum (MpAb 1, diluted 1:250); (**d**) binding with mouse polyclonal antiserum (MpAb 2, diluted 1:50).

chosen, since they were critical for binding to the antibodies from the replacement-net analysis, whilst Glu$^{288}$ was chosen as a control, since its effect on binding the antibodies was relatively neutral.

## Enzyme characterization

The mutant enzymes were purified by cation-exchange chromatography in a similar manner to the recombinant wild-type enzyme. High yields of purified enzyme were obtained for the enzymes P285T (64%), P2186Q (68%) and E288A (94%). The purified enzymes had subunit $M_r$ values of 35000, which were identical with that obtained for the wild-type enzyme. The subunit $M_r$ of the *E. coli* enzyme was also 35000. The pI values of the recombinant wild-type, P285T and P286Q enzymes were 8.6, and that for the E288A enzyme was 9.2. The pI of the *E. coli* enzyme was 4.9 and was similar to the reported value (Labourer et al., 1971).

## Steady-state kinetics

The kinetic constants of the mutant and recombinant wild-type enzymes with the substrates L-asparagine and L-glutamine are shown in Table 1. The kinetic constants of mutants P286Q and E288A with both substrates were comparable with that of the recombinant wild-type enzyme. The $k_{cat.}$ of mutant P285T with L-asparagine had been reduced to about half that of the wild-type enzyme, whilst $k_{cat.}$ for L-glutamine was reduced by 4-fold. This reduced $k_{cat.}$ value for asparagine is still higher than that of the *E. coli* enzyme and is unlikely to alter the therapeutic effectiveness. The reduced activity against glutamine may reduce the toxic side effects caused by administration of asparaginase. The structural constraints imposed by Pro$^{285}$ and Pro$^{286}$ were not crucial in stabilizing the enzyme for it to bind to its substrate, neither was removal of the negative charge at Glu$^{288}$. Although the mutations P285T, P286T and E288A were designed to mimic the sequence of the *E. coli* enzyme at these positions, none of the mutant enzymes had catalytic properties similar to the *E. coli* enzyme.

## Antigenic characterization

Binding of the mutant enzymes to antibodies raised against the wild-type enzyme was studied by sandwich e.l.i.s.a. (Figure 6). Lower binding compared with the wild-type enzyme indicated reduced antigenicity. The mutation P285T reduced the antigenicity of L-asparaginase by 8-fold compared with the wild-type enzyme, whilst P286Q and E288A reduced the antigenicity of L-asparaginase by 2- and 1.5-fold respectively. These results were typical for two monoclonal antibodies raised against the wild-type enzyme and was reproducible for a set of six duplicate experiments.

The X-ray crystal structure of *E. coli* L-asparaginase has been recently determined (Swain et al., 1993). It is likely that the *Er.*

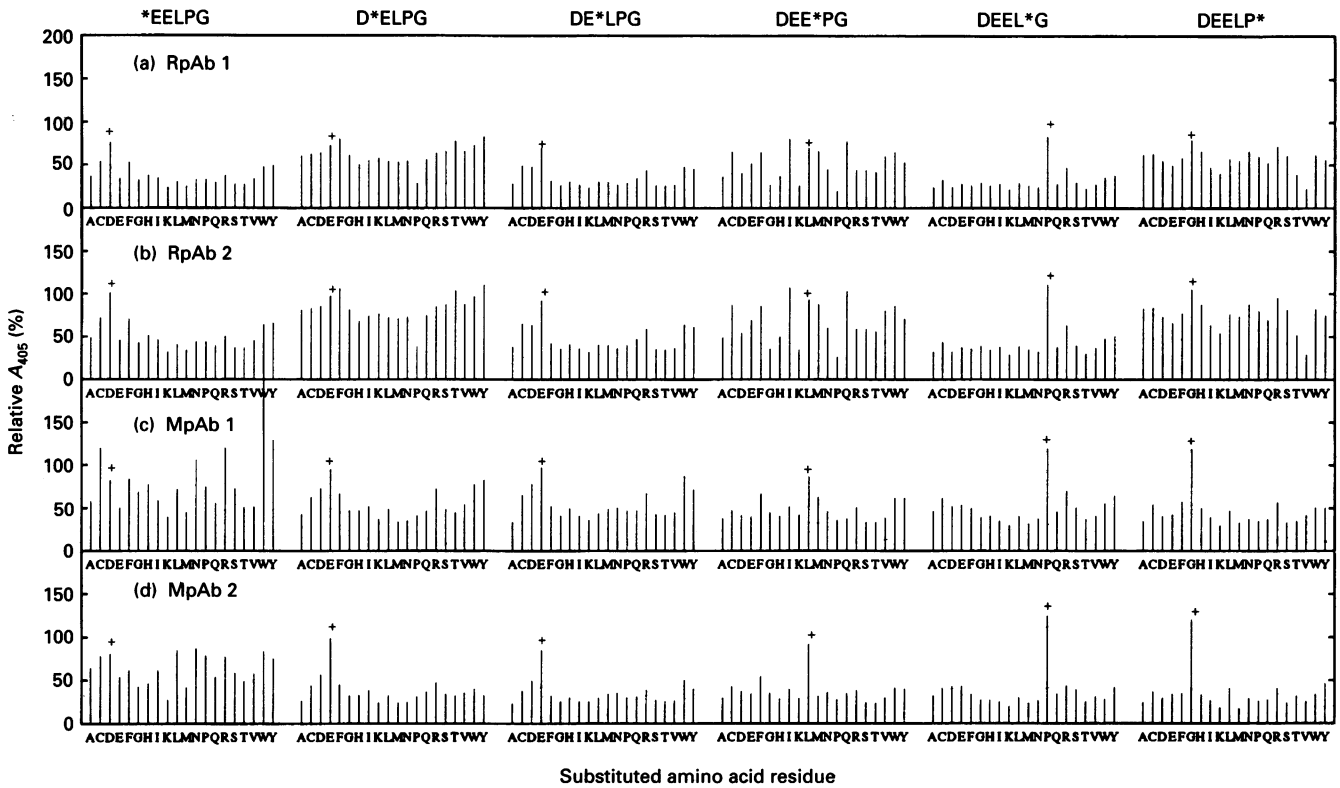


**Figure 5 Replacement-net analysis for the peptide $^{287}$DEELPG$^{292}$**

Antigenic peptides in the sequence $^{287}$DEELPG$^{292}$ with each of the amino acids replaced sequentially by one of the 20 amino acids from Ala to Tyr (the amino acid replacement is denoted by *). Results are of relative absorbance expressed as a percentage of that of the parent peptide (DEELPG) versus each of the peptides containing substituted amino acids. Binding is with polyclonal antiserum raised against native *Er. chrysanthemi* L-asparaginase. E.l.i.s.a. values for the six copies of the parent sequence have been averaged and taken as 100%. '+' denotes the position of the parent peptide. (**a**) Binding with rabbit polyclonal antiserum (RpAb 1, diluted 1:200); (**b**) binding with purified rabbit polyclonal antibodies (RpAb 2, 20 $\mu$g ml$^{-1}$); (**c**) binding with mouse polyclonal antiserum (MpAb 1, diluted 1:250); (**d**) binding with mouse polyclonal antiserum (MpAb 2, diluted 1:50).

**Table 1 teady-state kinetic constants for the recombinant wild-type (rec. wt), the mutant (mut) enzymes and *E. coli* L-asparaginase using L-asparagine and L-glutamine**

| Substrate... | L-Asparagine | | L-Glutamine | |
|---|---|---|---|---|
| Enzyme | $K_m$ ($\mu$M) | $k_{cat.}$ ($s^{-1}$) | $K_m$ ($\mu$M) | $k_{cat.}$ ($s^{-1}$) |
| rec. wt | 80±5 | 440±5 | 1670±170 | 65±3 |
| mut P285T | 90±6 | 170±2 | 1700±150 | 15±0.5 |
| mut P286Q | 95±5 | 380±4 | 1690±80 | 50±1 |
| mut E288A | 150±12 | 480±8 | 1880±120 | 60±2 |
| *E. coli* L-asparaginase | 50±5 | 100±1.5 | 8020±1500 | 5±1 |

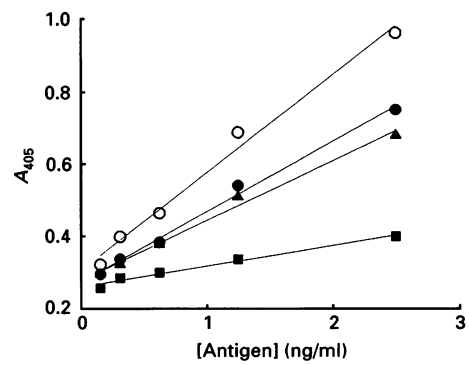


**Figure 6 Sandwich e.l.i.s.a.**

The Figure shows binding of purified monoclonal antiserum immobilized on the microtitre plate raised against *Er. chrysanthemi* L-asparaginase, with the wild-type and mutant enzymes (0.2–3.0 ng·ml$^{-1}$). The amount of antigen that bound was detected with rabbit polyclonal antibodies (diluted 1:500) raised against the native enzyme for further quantification. ○, Wild-type enzyme; ●, mutant E288A; ▲, mutant P286Q; ■, mutant P285T.

*chrysanthemi* enzyme is structurally similar, because of amino acid sequence similarities. Examination of the structure shows that the immunodominant region near the C-terminus is located at the surface of the enzyme, with a high relative flexibility, and would be expected to be more accessible for interaction with the antibody. Correlations between antigenicity and segmental mobility of antigenic regions on proteins show that antibodies raised against highly mobile regions bind strongly to the native protein, while antibodies to well ordered regions do not (Tainer et al., 1984).

The mutation P285T caused the greatest reduction in antigenicity of the enzyme, and may therefore be most useful in further studies aimed at evaluating the use of antigenically modified enzymes for therapy.

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