Published in final edited form as:

Protein Expr Purif. 2013 September ; 91(1): . doi:10.1016/j.pep.2013.06.010.

Expression and characterization of an enantioselective antigen-binding fragment directed against α -amino acids

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Abstract

This work describes the design and expression of a stereoselective Fab that possesses binding properties comparable to those displayed by the parent monoclonal antibody. Utilizing mRNA from hybridoma clones that secrete a stereoselective anti-L-amino acid antibody, a corresponding biotechnologically produced Fab was generated. For that, appropriate primers were designed based on extensive literature and databank searches. Using these primers in PCR resulted in successful amplification of the V_H, V_L, C_L and C_{H1} gene fragments. Overlap PCR was utilized to combine the V_H and C_{H1} sequences and the V_L and C_L sequences, respectively, to obtain the genes encoding the HC and LC fragments. These sequences were separately cloned into the pEXP5-CT/TOPO expression vector and used for transfection of BL21(DE3) cells. Separate expression of the two chains, followed by assembly in a refolding buffer, yielded an Fab that was demonstrated to bind to L-amino acids but not to recognize the corresponding D-enantiomers.

Keywords

Enantioselective antibody fragment; stereoselective Fab; expression; overlap extension PCR; mouse antibody constant regions

Introduction

Due to their unsurpassed binding properties, immunoglobulins are probably the most widely used affinity capture reagents. For decades, both polyclonal and monoclonal antibodies have been employed in, e.g., biochemical research, diagnostics, and therapeutic applications.[1–3] More recently, advances in molecular biological methods have opened up new opportunities for the biotechnological production and targeted manipulation of antibody-fragments, which have quickly gained even greater popularity for, e.g., the development of novel antibody-based drugs.[4,5]

As early as in 1928, Karl Landsteiner recognized that appropriately raised antibodies may exhibit a high degree of stereoselectivity. However, this discriminatory potential appears to have largely been neglected by the scientific community. One noteworthy exception is the

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field of catalytic antibodies where, starting in the 1980s, antibodies have been produced that can catalyze the conversion of suitable substrates with stereo- and regioselectivity.[6–8] Only few applications have utilized antibodies that stereoselectively bind to molecules in their ground state (as opposed to molecules in the transition-state, as is the case with catalytic antibodies). Such antibodies have mostly been used in radioimmunoassays for the detection of chiral drugs and their metabolites, respectively.[9] Only within the last fifteen years, stereoselective antibodies have become more popular tools for the analysis of chiral molecules.[10] For example, it has been demonstrated that stereoselective antibodies can be utilized for the ultra-sensitive detection of enantiomers in immunoassays [11, 12] and sensors, [13–19] as well as for chiral separation in chromatography.[20–28] With few exceptions, [24–26] these studies typically utilized antibodies that had been produced by classical immunological techniques and, thus, involved immunization of, e.g., rabbits or mice.

Since the biotechnological production of antibodies promises to reduce the need for laboratory animals, it is intriguing to use molecular biological techniques also for the generation of stereoselective antibody fragments. In addition, the availability of plasmids encoding the information for such antibodies not only opens the door to their targeted manipulation and mass production, but also facilitates further biophysical and structural biological characterization.

Immunoglobulin G (IgG) molecules are glycoproteins (MW ~150 kDa) composed of four polypeptide chains, namely two light chains (V_L - C_L) of about 25 kDa each, and two heavy chains (V_H - C_H1 - C_H2 - C_H3) of ca. 50 kDa each. The light and heavy chains are held together by non-covalent bonds and disulfide bridges between the C_L and C_H1 domains. An Fab fragment represents the antigen-binding arm of an antibody; it is composed of a light chain, LC, which consists of the V_L and C_L domains, and a heavy chain, HC, which comprises the V_H and C_H1 domains. Papain, a nonspecific thiol-endopeptidase, can be used to enzymatically cleave whole IgG molecules at the hinge region to yield two identical Fab fragments and one Fc fragment.[29–33]

The Fab fragments, which contain the complementarity determining regions (CDRs), are known to be more stable than molecular biologically produced single chain Fv (scFv) fragments.[34,35] It is also known that Fab exhibit binding properties similar to or indistinguishable from those of their whole parent antibody.[36,37] Due to their small size, rapid blood clearance, and low immunogenicity, Fab have been widely utilized in diagnostic, therapeutic, and research applications.[38,39] For example, Fab fragments have been employed in the treatment of digoxin ingestion,[40] snake bites,[41] *Entamoeba histolytica*,[42] rabies,[43] thrombosis, and tumors.[44]

Here, we describe the construction and expression of a stereoselective Fab fragment in *E. coli* using the genetic material of hybridoma cells that produce an antibody that stereoselectively binds to the L-enantiomers of α -amino acids but not to the corresponding D-enantiomers.[28,45,46] Similar parent antibodies have successfully been used for enantiomer detection and separation in a variety of analytical techniques.[11–17,20–23,27,28,45,46]

Experimental

Production and characterization of the parent antibody

The monoclonal anti-L-amino acid antibody 80.2 (anti-L-AA 80.2) was produced with permission of the Institutional Animal Care and Use Committee at Northern Illinois University (ORC #292) following previously published procedures.[45]

Antibody stereoselectivity was first verified in noncompetitive ELISAs on 96-well microtiter plates (Nunc, Rochester, NY) using three different solid-phase coatings, namely either underivatized BSA, or the conjugates of bovine serum albumin (BSA) and *p*-amino-D- or -L-phenylalanine, prepared by diazotization. Competitive ELISAs were performed utilizing the BSA-conjugate of *p*-amino-L-phenylalanine as coating and the pure enantiomers of various α -amino acids as inhibitors.

Sequence analysis of monoclonal antibody anti-L-AA 80.2

The mRNA of 10^8 hybridoma cells producing anti-L-AA 80.2 was isolated on an oligo(dT) spin column (Qiagen, Valencia, CA) and converted into cDNA in a first-strand reaction with reverse transcriptase using the primer first-strand mix kit (Amersham Pharmacia Biotech, Little Chalfont, England). For the amplification of antibody heavy and light chain DNA (~340 and ~325 bp, respectively), the degenerate primers described by Wang et al. [47] were used. Heavy and light chain DNA was ligated into the plasmid pCR4.1 for transfection into *E. coli* Top 10 (Invitrogen, Carlsbad, CA). Sequencing reactions were carried out by Dr. Scott Grayburn at the Core DNA Synthesis and Sequencing Facility at Northern Illinois University using a Beckman CEQ8000XL Capillary System (Beckman Coulter; Fullerton, CA).

The nucleotide sequences were analyzed using Sequencher,[48] and converted into the corresponding amino acid sequences using the translation tool on the ExPASy Server.[49] The program Basic Local Alignment Search Tool (BLAST; ref. 50) was utilized to verify the origin of the sequences by comparing them with sequences in the National Center for Biotechnology Information's (NCBI) non-redundant (nr) database. The sequences were aligned with mouse heavy or light chain variable regions compiled in the Kabat database [51,52] to determine positions of the framework and hypervariable regions.

Production of stereoselective antibody fragments by molecular biological techniques Amplification of V_H and V_L fragments

Based on sequence analysis of the monoclonal antibody 80.2, two sets of primers, 80.2VH_fw (5'-GAGGTCCAGCTGCAACAATCAGG-3') and 80.2VH_rev (5'-GAGAGACATGACCAGAGTCCCTTGG-3') for V_H, and 80.2VL_fw (5'-GACATTGTGCTGACCCAATCTCCA-3') and 80.2VL_rev (5'-GTTTCGCTCCAGCTTGGTCCCA) for V_L were designed and used to amplify the variable sequences of heavy and light chains from cDNA by PCR. Reaction mixtures were composed of 5 μ L of 10x *Taq* buffer containing Mg²⁺ (Eppendorf, Hamburg, Germany), 1 μ L of 10 mM deoxynucleotide triphosphates (Promega, Madison, WI) at a final concentration of 0.2 mM, 1 μ L of forward and reverse primers at a final concentration of 0.1–0.2 μ M, 1–150 ng/ μ L template DNA, 0.4–1 μ L (corresponds to 0.5–2.5 units) of *Taq* DNA polymerase (Eppendorf, Hamburg, Germany), and nuclease-free water to yield a total volume of 50 μ L. The thermocycler program was as follows: 1 cycle at 94 °C for 3 min, followed by 30 cycles (94 °C for 1 min, 55 °C for 1 min, and 72 °C for 2 min) and a final cycle of 10 min at 72 °C. Amplified DNA was analyzed in agarose gels and purified using the Wizard SV Gel and PCR Clean-Up System (Promega, Madison, WI).

Construction of the mouse heavy constant chain (C_H1) and the mouse kappa constant chain (C_L)

In order to identify appropriate primers for the amplification of the constant regions, several kappa light chain constant regions (C_L) and heavy chain constant regions from different sources were compared and evaluated. For the production of the C_L domain, the sequence MOPC 21 # 8 from the Kabat database [53] was used for designing the forward and reverse primers: CL_fw (5'-GCTGATGCTGCACCAACTGTATCCATCTTCCCACC-3') and

CL_rev (5'-ACACTCATTCTGTGAAGCTCTTGACAATGG-3'). The C_H1 nucleotide sequence from BC024405.1 (from BLAST) was selected as template for the design of the primers CH1_fw_constant (5'-CCAAAACGACACCCCATCTGTCTATCCACT-3') and CH1_rev_constant (5'-ACCACAATCCCTGGGCACAATTTCTTGTCCACC-3'), which were used to amplify the C_H1 sequence from 80.2 cDNA.

The PCR conditions for the amplification of the constant regions were the same as described above for the variable regions.

Assembly of the 80.2 HC and LC fragments

To allow generation of the whole 80.2 HC sequence by PCR, the two fragments, 80.2 V_H and 80.2 C_H1, should ideally overlap by 15–35 bp. The forward primer, CHextension80.2VH: 5'-CTGGGGCCAAGGGACTCTGGTCATGTCTCTCGCCAAAACGACACCCCATCTGTCTATCCACT-3', was designed, which contains the antisense of the 80.2 V_H 3' end at its 5' end (represented by the underlined nucleotides).

Using the above described PCR conditions, an overlap with 80.2 V_H was created at the 3' start of 80.2 C_H1 resulting in an extended 80.2 C_H1.

For the 80.2 LC sequence, the primer CL.o/v.80.2VL.: 5'-TGGGACCAAGCTGGAGCGAAACGCTGATGCTGCACCAACTGTATCCATCTTC-3' was designed with a 20 bp overhang from the 3' end of the 80.2 V_L complement region. This produced an extended 80.2 C_L sequence.

Overlap extension PCR was then carried out for the creation of the heavy chain (HC) and light chain (LC) fragments, using V_H and extended C_H1 and V_L and extended C_L sequences, respectively. Primers utilized for HC were 80.2VH_fw and CH1_rev_constant, while the primers for LC were 80.2VL_fw and CL_rev_constant. PCR was performed using 5 µL of 10x *Taq* buffer, 1 µL of the appropriate forward and reverse primers, 5 µL of appropriate DNA templates, 2.5 µL dNTPs, 5 µL MgCl₂ (at 25 mM), 0.7–1.0 µL (0.5–2.5 units) *Taq* DNA polymerase, and sterile water to obtain a final volume of 50 µL. PCR conditions were as follows: 1 cycle at 94 °C for 3 min, followed by 30 cycles (94 °C for 1 min, 55 °C for 2 min, and 72 °C for 2 min) and a final extension for 10 min at 72 °C. PCR products were purified from agarose gels using the Wizard SV Gel and PCR Clean-Up System.

Expression of the Fab protein

HC and LC fragment DNA sequences, respectively, were ligated into the pEXP5-CT/TOPO cloning vector using a TOPO cloning reaction. DNA was analyzed in agarose gels and purified using the Wizard SV Gel and PCR Clean-Up System prior to electroporation and transformation into *E. coli* BL21 DE3. The 80.2 HC and 80.2 LC antibody proteins were expressed in separate cultures. Cells in LB (5 mL) containing ampicillin (100 µg/mL) were inoculated with both vectors and 1 L cultures were grown overnight (37 °C; 250 rpm) to an OD₆₀₀ of 0.5–0.7. Each cell culture was then induced with IPTG (1 mM final concentration) and incubated for 4 h (37 °C, 250 rpm).

The cells were harvested by centrifugation at 3,200 rpm for 20 min at 4 °C. Extraction of the recombinant protein from the BL21(DE3) *E. coli* cells was performed using the B-PER II Bacterial Protein Extraction Reagent (Thermo Fisher, Rockford, IL) according to the manufacturer's instructions. Other lysis buffers were investigated but did not show any improvement in yield. The total amount of secreted protein in bacterial culture supernatants was very low. To increase protein yield, cell pellets from 1 L bacterial culture were resuspended with 30–50 mL B-PER (between 15 and 25 g wet cells were obtained from a 1

L culture). After incubation for 15 min cell debris was removed by centrifugation at 13,000 rpm for 5 min. Whole cell lysates from 1 L culture were then suspended in 50 mL of unfolding buffer consisting of 6 M guanidine-HCl, 50 mM NaH₂PO₄, 300 mM NaCl, 2 mM reduced glutathione (GSH), and 0.2 mM oxidized glutathione (GSSG), pH 7.4, and stirred for 24–36 h at 4 °C. Centrifugation (15 min at 8000 rpm) was used to remove any insoluble material.[54]

Different induction times (0 to 16 h) were investigated to optimize protein expression. For this experiment, both fragments were His-tagged. Protein samples containing His-tagged HC and LC, respectively, were dialyzed against PBS and purified on Talon Metal Affinity resin (Clontech Laboratories, Mountain View, CA). Depending on the amount of protein to be purified, column bed volumes were either 5 or 50 mL with a capacity of 3 mg of protein per 2 mL of gel. In brief, the talon resin was resuspended, transferred to a sterile tube and centrifuged at 700 x g for 2 min to pellet the resin. The supernatant was removed and discarded. The resin was then briefly mixed with 10 bed volumes of equilibration/wash buffer (50 mM Na₃PO₄, 300 mM NaCl, pH 7.0) and pre-equilibrated before recentrifugation at 700 x g for 2 min. The equilibration/wash step was repeated two more times. Then, the protein sample was added to the resin and the mixture was agitated at room temperature for 20 min on a platform shaker to allow the His-tagged protein to bind to the resin. The mixture was centrifuged at 700 x g for 5 min and the supernatant was carefully removed. The pellet was resuspended in 10 – 20 bed volumes of equilibration/wash buffer and gently agitated at room temperature for 10 min on a platform shaker. The suspension was then centrifuged at 700 x g for 5 min and the supernatant removed and discarded. This washing step was repeated two more times. Then, one bed volume of equilibration/wash buffer was added and the resin resuspended by vortexing. The resin was transferred to a gravity flow column with an end-cap in place and allowed to settle out of suspension. The endcap was removed and the buffer allowed to drain until it reached the top of the resin bed. The column was washed once with 5 bed volumes of equilibration/wash buffer. His-tagged protein was eluted by adding 5 bed volumes of imidazole elution buffer (50 mM Na₃PO₄, 300 mM NaCl and 150 mM imidazole, pH 7.0) to the column. Eluate was collected in 500 µL fractions. The protein content of all fractions was determined by measuring the UV absorbance at 280 nm. Protein containing fractions were pooled and concentrated using Amicon Pro 10K centrifugal concentrators (EMD Millipore, Billerica, MA). Protein concentrations were determined using a NanoDrop® ND-1000 UV/Vis (Wilmington, DE, USA; Table 1). Spectra were recorded to ensure that no contamination from DNA/RNA was observed. For the assembly of the Fab, expression was carried out as described above but the LC fragment was not His-tagged to allow for purification of assembled Fabs. Solutions in unfolding buffer containing His-tagged HC and native LC fragments were mixed in a 1:1 ratio in order to allow heavy and light chains to associate. The resulting solution was diluted 1:10 with refolding buffer containing 20 mM Tris-HCl (pH 7.4), 1.0 mM EDTA, 2.0 mM GSH, and 0.2 mM GSSG. The mixture was gently stirred for 2 days at 4 °C to facilitate correct folding of protein. The protein solution was then dialyzed overnight against PBS (pH 7.4) at 4 °C.

Purification of His-tagged Fab was carried out on Talon Metal Affinity resin as described above for LC and HC fragments and samples were concentrated using Amicon Centrplus 30K centrifugal concentrators (EMD Millipore, Billerica, MA). Purified sample Fab fragments were analyzed by Western Blot and protein concentrations were determined using a NanoDrop® ND-1000 UV/Vis Spectrophotometer.

Characterization of Fab fragments

Expressed Fabs were tested by Dot blot and ELISA. The percentage of active protein was estimated utilizing affinity chromatography.

Dot blot: 2 μ L samples (at 1 mg/mL) were applied to a nitrocellulose membrane (Protran® pure nitrocellulose membrane, Schleicher & Schuell Inc., Keene, NH). Dots were dried at room temperature for about 1 h. After washing the membrane several times with PBS containing 0.05% (v/v) Tween 20 (PBS/Tween 20), unoccupied sites on the membrane were blocked by immersing it in PBS/Tween 20 containing 1% gelatin for 1 h at room temperature. The membrane was then washed multiple times with PBS/Tween 20, followed by incubation in a 1/5,000 dilution of horseradish peroxidase (HRP) labeled-secondary antibody (Jackson ImmunoResearch Laboratories, West Grove, PA) in PBS (1 h at room temperature). After washing the membrane again several times with PBS/Tween 20, tetramethyl benzidine (TBS; Endogen, MA, USA) in the presence of 13 μ L of 50 % hydrogen peroxide was added as substrate for color development. The blotted membrane was rinsed in water to stop the reaction.

Affinity chromatography: A stationary phase was produced that comprised an azo-structure analogous to the one contained in the hapten-carrier conjugate that was used in the production of the parent antibody. For that, 14 g Sepharose CL4B(wet weight) were washed successively with 1:3 (v/v) acetone:water, 1:1 (v/v) acetone:water, 3:1 (v/v) acetone:water, and dry acetone. The support material was then activated with a solution of 1.65 g of disuccinimidyl carbonate (DSC) and 1.35 g of dimethylaminopyridine (DMAP) dissolved in 20 mL of DMSO following the procedure by Wilchek and Miron.[55] The reaction was allowed to proceed overnight at 4 °C under gentle agitation. The activated stationary phase was successively washed with dry acetone, 5% acetic acid in 1,4, dioxane, methanol and finally isopropyl alcohol. The number of active groups was determined spectrophotometrically at 261 nm using a 1/10 dilution of a 4 mg aliquot of the Sepharose that had been hydrolyzed using 1 mL of 0.25 M ammonium hydroxide. Next, 200 mg of tyramine in 30 mL of PBS was reacted with the activated support material for 4 h at 4 °C. The stationary phase was then washed successively with PBS, 100 mM Tris buffer (pH 7.0), PBS, 1M NaCl and again PBS. Then, 20 mg *p*-amino-L-phenylalanine was dissolved in 1 mL of HCl, and an equimolar amount of sodium nitrate was added. After 10 min, the reaction mixture was slowly added to the suspension of stationary phase. Production of an azo-structure was obvious from a change in color to orange-red. The pH was adjusted to 8.5 with 0.1 M NaOH and the reaction allowed to proceed overnight at 4 °C. The prepared stationary phase was washed extensively with PBS, NaCl, triethylamine (TEA), citric acid, HCl and guanidine-HCl. Following each washing, the stationary phase was rinsed with water. 4.2 mg of the Fab was incubated with the affinity stationary phase for 2 hours at room temperature and then poured into a 20 mL glass column. The flow-through was collected and the column was washed first with PBS containing 1 M NaCl and then with PBS until no UV-absorbance at 280 nm was detected. Antibody was eluted utilizing 10 mM TEA (pH 12), and 1 mL fractions were collected and immediately neutralized with 27 μ L of 100 mM citric acid. Fractions with significant protein levels (as determined by measuring the UV-absorbance at 280 nm) were combined and dialyzed against PBS. Purified protein solutions were concentrated using Amicon Pro 10K centrifugal concentrators (EMD Millipore, Billerica, MA). Protein concentrations were determined using a NanoDrop® ND-1000 UV/Vis (Wilmington, DE, USA). A detailed description of the procedure can be found in reference 56. 4 mg of functional Fab was recovered from the column. This means that about 95% of the protein recovered from the Talon IMAC column were active. It should be noted that the binding capacity of the support material was no limiting factor in this experiment as it is significantly higher than 4 mg of antibody.

ELISA: 96-well Maxisorp polystyrene microtiter plates (Nunc, Rochester, NY) were coated with 100 μ L/well of a 1 μ g/mL solution of either the BSA-conjugates of *p*-amino-D-phenylalanine (*p*-azo-D-Phe-BSA) or *p*-amino-L-phenylalanine (*p*-azo-L-Phe-BSA), or underivatized BSA in 50 mM carbonate buffer, pH 9.6. After overnight incubation at 4 °C,

plates were washed three times with PBS/Tween 20. Unoccupied sites on the plate surface were blocked using 250 μ L per well of PBS/Tween 20 containing 1% gelatin. Plates were then incubated for 2 h at 37 °C, followed by three washes with PBS/Tween 20.

In noncompetitive tests, serial dilutions of Fab in PBS (100 μ L/well) were applied to the plate and incubated for 2 h at 37 °C. After washing three times as described above, goat anti-mouse F(ab')₂ secondary antibody labeled with HRP was added (1/5,000 in PBS; 100 μ L/well). Following incubation for 2 h at 37 °C and another washing step, the substrate *o*-phenylenediamine (Sigma, St. Louis, MO; 100 μ L/well) was added. Absorbance at 492 nm was read in a FLUOstar plate reader (BMG Labtechnologies, Offenburg, Germany) following acidification with 1 N sulfuric acid (100 μ L/well).

In competitive ELISAs, the coating, blocking, and washing steps were performed as described above. However, primary antibody, i.e., Fab, was applied at a constant concentration (50 μ L/well in PBS) and incubated (2 h at 37 °C) in the presence of competitors at varying concentrations (50 μ L/well). As competitors, the pure enantiomers of a number of free amino acids were used. Absorbance values were converted into % inhibition using the equation: % inhibition = $[1 - (A/A_0)] \times 100$, where A represents values in the presence of competitor, while A₀ is the absorbance measured in the absence of competitor. All tests were performed at least in triplicate.

Results and Discussion

In this study, enantioselective Fab fragments were generated utilizing the mRNA of the monoclonal anti-L-amino acid antibody 80.2 (anti-L-AA 80.2) that had been produced previously following classical immunological methods. [45,46,57]

The DNA sequence of the anti-L-amino acid antibody 80.2 was cloned into the pEXP5-CT/TOPO expression vector, and the expressed protein was characterized using SDS-PAGE, ELISA, western blot, and dot blot analysis.

Sequence analysis of the parent antibody

In order to amplify the complementary DNA encoding the variable regions of the heavy and light chains of anti-L-AA 80.2, degenerate primers targeting the framework region one (FR1) and the beginning of the constant region (C1) were employed in a polymerase chain reaction (PCR).[47] Following sequence analysis and conversion into the corresponding amino acid sequence, the V_H and V_L sequences shown in Figure 1 were obtained.

The Basic Local Alignment Search Tool (BLAST) was used to compare the nucleotide and amino acid sequences of V_H and V_L of anti-L-AA 80.2 (80.2 V_H and V_L) with those contained in the GenBank database.[58] The results from the BLAST search database confirmed that both 80.2 V_H and V_L are from mouse (*Mus musculus*); the highest score (Bits) for the 80.2 V_H was 466 with an E-value of 7e-129, while the highest score (Bits) for 80.2 V_L was 509 with an E-value of 5e-142.

Production of stereoselective antibody fragments by molecular biological techniques

The method for constructing Fab antibodies consists of the following: (1) Primary PCR amplification of V_H, V_L, C_L and C_{H1} gene fragments from 80.2 hybridoma cDNA; (2) Joining of the V_H and C_{H1}, and V_L and C_L sequences via overlap PCR to create HC and LC fragments, respectively; (3) Cloning of the HC and LC gene fragments into separate pEXP5-CT/TOPO expression vectors; (4) Electroporation, growth and expression of the recombinant vectors in BL21(DE3) cells.

Based on the sequence analysis of the parent antibody, primers were designed to amplify the DNA sequences of 80.2 V_H and V_L by PCR (see section Experimental). Analysis of the PCR products in a 0.9 % agarose gel showed bands for the V_H and V_L DNA sequences at about 385 bp and 350 bp, respectively, which is in agreement with the expected sizes.

Since reliable mouse constant region primers could not be found in the literature, and C_H1 is highly conserved, a number of previously published mouse heavy chain sequences were compared. These included oligonucleotides from murine constant region C_H1 primers,[59–61] amino acid and nucleotide sequences from various murine C_H1 fragments,[62–64] and the DNA sequence IGG1 #37 from the Kabat database.[53] A BLAST search confirmed that all these sequences originated from murine IgG antibody. The sequence IGG1 #37 is almost identical with the mouse antibody sequence BC024405.1 of the BLAST database but lacks about 18 nucleotides at the 3' end. Because of the high degree of homology of the variable domains of 80.2 and BC024405.1 the C_H1 nucleotide sequence of the latter was selected as template for the design of the primers CH1_fw_constant and CH1_rev_constant, which were used for amplifying the C_H1 sequence of anti-L-AA 80.2. Agarose gel electrophoresis of the PCR products showed the heavy constant fragment at about 359 bp, which is in agreement with the expected size. The DNA encoding the C_H1 gene was confirmed by sequencing, and a BLAST search verified that the sequence (Figure 2a) was consistent with mouse C_H1 with a Bits score of 579 and an E-value of 6e–163.

A similar strategy was applied to obtain mouse light chain constant region (C_L) primers. Several published sequences were compared, namely a complete murine kappa chain constant sequence, oligonucleotides from murine C_L primers,[59,60,64] amino acid and nucleotide sequences from various murine C_L fragments,[65,66] and the sequence MOPC 21 # 8 from the Kabat database.[53] Among the query sequences, MOPC 21 # 8 resulted in the best match with the mouse C_L sequence AB048524 (RefSeq ID); the highest score (Bits) was 588 and the E-value was 1e–165. Alignment between MOPC 21 # 8 and AB048524.1 showed that the sequences are in fact identical, which again is not surprising since also C_L is highly conserved. The MOPC 21 # 8 sequence was therefore used to design the primers CL_fw and CL_rev. Using these primers and cDNA of anti-L-AA 80.2, the C_L region could successfully be amplified by PCR yielding a DNA product of about 321 bp, which was subsequently gel purified and sequenced (see Figure 2b).

Construction of the heavy chain gene fragment (HC) was carried out by overlap extension PCR. To allow generation of the whole 80.2 HC sequence by PCR, the two fragments, 80.2 V_H and 80.2 C_H1, should ideally overlap by 15–35 bp. For this reason, the following forward primer, CHextension80.2VH, was designed which contains the antisense of the 80.2 V_H 3' end at its 5' end. The 80.2 C_H1 DNA was then amplified and the 33 nucleotides of 80.2 V_H were added to the 5' end of the 80.2 C_H1 sequence. As a consequence, the 5' end of 80.2 C_H1 and the 3' end of 80.2 V_H hybridized during the overlap PCR to form a complete 80.2 HC monocistronic gene fragment (Figure 3) with a length of about 670 bp.

The produced 80.2 HC DNA fragment was sequenced in its entirety at the Core DNA Analysis Lab at Northern Illinois University (Figure 4a).

Similarly to the HC fragment, the 80.2 LC fragment was constructed by joining V_L and C_L in an overlap extension PCR. Primer CL.o/v.80.2VL was designed with a 20 bp overhang complementary to the 3' end of the 80.2 V_L complement region. Upon PCR amplification, a complete 80.2 LC gene of approximately 645 bp consisting of V_L and C_L was obtained and sequenced (Figure 4b) to confirm the open reading frame.

Making use of the terminal transferase activity of *Taq* polymerase, amplification of 80.2 HC and LC fragment DNA added single deoxyadenylate (A) residues to the 3' ends of the PCR

products. These were utilized in TOPO cloning reactions to form A–T hybrids with the expression vector. In addition, new forward primers were designed containing overhang nucleotides, “CCATG”, to be incorporated at the 5' ends of both 80.2 HC and LC DNA fragments. Addition of the start codon “ATG” to the overhang was necessary to facilitate proper translation of the recombinant protein. The “CC” was incorporated to ensure proper spacing between the ribosome binding site and the initial ATG codon for the desired protein.

Since LC fragments have a tendency to form homo-dimers, known as Bence Jones proteins, [67,68] a reverse primer was designed to contain a premature stop codon at the 3' end preventing protein translation beyond the LC fragment. As a consequence, the LC protein would be free of an incorporated His-tag at the C-terminus, which is part of the pEXP5-CT/TOPO plasmid. This approach was intended to prevent co-purification of LC protein or LC homo-dimers along with correctly associated Fab antibody on a metal affinity column.

The pEXP5-CT/TOPO recombinant vectors containing the 80.2 HC and LC gene fragments, respectively, were separately transformed into BL21(DE3) *E. coli* cells, which facilitates high levels of protein expression due to the tight control of the T7 promoter. Transformed cells were screened for the presence and correct orientation of the HC and LC genes; this was necessary as TOPO cloning is non-directional due to the T-A hybridization.

Both the 80.2 HC and 80.2 LC antibody proteins were expressed separately. A positive colony for each fragment was used to inoculate an overnight culture. Protein expression was induced with IPTG and whole cell lysates were extracted using the B-PER II Bacterial Protein Extraction Reagent and unfolding/refolding buffer as described in Experimental, section “*Expression of the Fab protein.*” SDS-PAGE gels showed no difference in the amount of protein obtained upon cell disruption using B-PER II Bacterial Protein Extraction Reagent or other lysis buffers (results not shown), but sonication lead to a significant decrease in the amount of protein. Therefore, B-PER II Bacterial Protein Extraction Reagent was used for protein extraction. The 80.2 HC and LC fragments both showed bands at approximately 25 kDa (Figure 5, lanes 1 and 2).

In order to optimize the induction time for the production of the protein fragments, expression yields for HC and LC obtained after different times were compared. It was found that induction after 4 h gave the highest yield of IMAC-purified protein for both fragments (Table 1). Since the expression vector was void of a signal sequence, protein should have been retained in the cytoplasm. The basal level of protein expression is likely due to a leaky plasmid, but expression levels could be improved upon induction with IPTG.

Following insertion of the HC and LC sequences into expression vectors, both the 80.2 HC and 80.2 LC antibody fragments were expressed separately and isolated from whole cells using B-PER and unfolding buffer [69,70]. They were then mixed at an equal molar ratio in refolding buffer. It was assumed that the individual fragments would find each other to produce a whole functional 80.2 Fab protein fragment. Antibody formation was investigated by dot blot. As seen in Figure 6, colored dots were only observed with refolded samples or positive controls, while neither non-refolded samples nor the lysis products of non-transformed BL21(DE3) cells used as negative control caused color formation. This result indicated that a properly assembled and folded Fab fragment had been formed using the protocol previously described for VHH antibodies,[71] which are single domain antibodies engineered from heavy-chain antibodies found in camelids.[71]

The Fab was designed to contain a His-tag at the C-terminus of the heavy chain for purification on a metal affinity resin. Following the Talon Metal Affinity Resin protocol, protein-containing fractions were collected and further analyzed by SDS-PAGE. Bands obtained under non-reducing conditions at the expected MW of 50–55 kDa indicated that the

80.2 HC and LC fragments had indeed successfully refolded and combined to form an Fab (Figure 5, lane 4 and 5). An additional band at 25 kDa can be attributed to HC which coeluted with the Fab, as 80.2 HC was expressed with a His-tag. As expected, the Fab dissociated into HC and LC fragments under reducing conditions (Figure 5, lane 7).

The amount of properly folded, active 80.2 Fab that could be recovered from a 1 L culture was approximately 4 mg as estimated by affinity chromatography on an immobilized derivative of *p*-amino-L-phenylalanine following a previously published procedure.[56] 95 % of the protein eluted from the IMAC column was active. Considering the amounts of LC and HC obtained after 4 h of induction (Table 1), this corresponds to a yield of about 1%. Western blot analysis, furthermore, showed that a secondary anti-mouse antibody was able to recognize the expressed protein following non-reducing gel conditions (not shown).

Evaluation of the binding specificity of produced antibody fragments

Functionality as well as stereoselectivity of the purified 80.2 Fab antibody was first assessed using noncompetitive ELISA. The 80.2 Fab stereoselectively bound to the BSA conjugate of the hapten *p*-amino-L-phenylalanine (p-azo-L-Phe-BSA) that had been used as solid phase coating, but no binding to the corresponding conjugate of the D-enantiomer or underivatized BSA was observed (Figure 7). This is in agreement with the binding properties displayed by the parent -L-amino acid antibody 80.2.[72]

The interaction between antibodies and antigens is governed by amino acid residues in the hypervariable regions within both the V_H and V_L. In order to examine the importance of each of the two polypeptide chains on the stereoselectivity and antigen-binding capacity of the 80.2 Fab antibody, HC and LC were also expressed, purified, and tested separately. For this purpose, a His-tag was incorporated into the LC DNA sequence to facilitate purification of the 80.2 LC protein by IMAC. Gel analysis following expression and purification showed bands corresponding to purified HC and LC antibody fragments, respectively (not shown). ELISA experiments performed with the HC and LC individual chains did not indicate specific binding activity or stereoselectivity of the individual fragments, which implies that only the combination of both yields a functional protein.

The assembled 80.2 Fab antibody protein was then further characterized in competitive ELISAs using the enantiomers of various amino acids as inhibitors. In this type of experiment, free amino acids in solution compete with the antigen-coating on the microtiter plate for the antibody's binding site. The concentration of an applied competitor needed to inhibit antibody binding to the immobilized antigen correlates with the antibody's affinity for this compound. That is, if the affinity is high, a relatively low concentration of the competitor will be sufficient to decrease antibody binding to the solid-phase immobilized antigen. Similarly, if the affinity is weak, a higher concentration of competitor is required.

ELISA results individually obtained with the D- or L-enantiomers of the amino acids phenylalanine, *p*-amino-phenylalanine, tryptophan, and cyclohexylalanine are shown in Figure 8.

The relative binding affinities of the Fab antibody were deduced from the IC₅₀ values (Table 2). These values represent the concentration of competitor resulting in a 50 % inhibition of antibody-binding to immobilized antigen. Since the original antibody, anti-L-amino acid antibody 80.2, had been raised against an immunogen displaying a phenylalanine-based azo-structure on its surface, it was expected that the Fab would bind more strongly to competitors that closely resemble the hapten. Thus, it is not surprising that the lowest IC₅₀ values were obtained with L-phenylalanine and *p*-amino-L-phenylalanine (Table 2).

Although L-cyclohexylalanine and L-tryptophan are bound more weakly by the Fab as indicated by their higher IC₅₀ values, it should be noted that also the interaction of the antibody with these compounds is highly stereoselective. The obtained results clearly show that the Fab only binds to amino acids having the correct (i.e., L-) configuration. In addition, L-amino acids containing different side chains can be bound, albeit with varying affinities. All these observations are in agreement with previous results obtained with the parent antibody 80.2 and similar antibodies.[45,46]

It is noteworthy that the overall low affinity observed for the interaction between such antibodies and L-amino acids is no impediment for their use in analytical applications, but may even be desirable, e.g., for enantiomer separation under isocratic elution conditions.[20]

Availability of biotechnologically produced stereoselective antigen-binding fragments is expected to greatly facilitate their utilization in analytical techniques and the structural elucidation of the molecular basis of their chiral discrimination properties.

Acknowledgments

This work was supported by the National Institutes of Health (1 R15 GM076000-01). We would like to thank Dr. Scott Grayburn from the Core DNA Analysis Lab at NIU for sequencing.

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Highlights

- Design and expression of a stereoselective Fab
- Amplification of variable heavy and light chain domains from mRNA of parent monoclonal antibody
- Construction of constant domains based on database searches
- Overlap extension PCR to produce Fab
- Fab binding properties comparable to those displayed by the whole parent monoclonal antibody

80.2 V_H:

EVQLQQSGGGLVKPGGSLKLSAASGFTF**SNYDTS**WVRQTPEKRLEWVT**SIS**SGGYTYYPDSV
KGRFTISRDNARNILYLQMSSLRSEDTAMYYCAR**ALYDYTTSPWFAY**WGQGLVMSL

80.2 V_L:

DIVLTQSPAIMASAPGEKVTMT**CRASSSVSSSYLH**WYQQKSGASPKLWIY**STSNLAS**GVPARFS
GSGSGTSYSLTISSVEAEDAATYYC**QQYSGTPLT**FGAGTKLERN

Figure 1.

Amino acid sequences of the V_H and V_L domains of anti-L-AA 80.2.

(a) 80.2 C_{H1}:

GCCAAAACGACACCCCCATCTGTCTATCCACTGGCCCCCTGGATCTGCTGCCCAAACCTAACTCCA
TGGTGACCCTGGGATGCCTGGTCAAGGGCTATTTCCCTGAGCCAGTGACAGTGACCTGGAACCTC
TGGATCCCTGTCCAGCGGTGTGCACACCTTCCCAGCTGTCCTGCAGTCTGACCTCTACACTCTG
AGCAGCTCAGTGACTGTCCCCTCCAGCACCTGGCCCAGCCAGACCGTCACCTGCAACGTTGCCC
ACCCGGCCAGCAGCACCAAGGTGGACAAGAAAATTGTGCCCAGGGATTGTGGC

5'3' Frame 1:

AKTTPPSVYPLAPGSAAQTNSMVTLGCLVKGYFPEPVTVTWNSGSLSSGVHTFPAVLQSDLYTL
SSSVTVPSSTWPSQTVTCNV AHPASSTKVDDKIVPRDCG

(b) 80.2 C_L:

GCTGATGCTGCACCAACTGTATCCATCTTCCCACCATCCAGTGAGCAGTTAACATCTGGAGGTG
CCTCAGTCGTGTGCTTCTTGAACAACCTTACCCCCAAAGACATCAATGTCAAGTGGAAGATTGA
TGGCAGTGAACGACAAAATGGCGTCCTGAACAGTTGGACTGATCAGGACAGCAAAGACAGCACC
TACAGCATGAGCAGCACCCCTCACGTTGACCAAGGACGAGTATGAACGACATAACAGCTATACCT
GTGAGGCCACTCACAAGACATCAACTTCACCCATTGTCAAGAGCTTCAACAGGAATGAGTGTGG
C

5'3' Frame: 1

ADAAPTVSIFPPSSEQLTSGGASVVCFLNNFYPKDINVKWKIDGSERQNGVLNSWTDQDSKDST
YSMSSTLTTLTKDEYERHNSYTCEATHKTSTSPIVKSFNRNECG

Figure 2.

Nucleotide and amino acid sequences of (a) 80.2 C_{H1} and (b) 80.2 C_L.

Figure 3.

PCR reactions used for the assembly of 80.2 HC. $80.2 V_H$ and $80.2 C_H1$ gene segments were first produced separately using cDNA from hybridoma cells. Overlap areas (shaded) complimentary to the end of $80.2 V_H$ were created at the beginning of C_H1 segments, resulting in an extended C_H1 (C_H1e). Overlap extension PCR using primers A and B yielded the complete 80.2 HC sequence.

(a) 80.2 HC:

GAGGTCCAGCTGCAACAATCAGGGGGAGGCTTAGTGAAGCCTGGAGGGTCCCTGAAACTCTCCT
GCGCAGCCTCTGGATTCACTTTCAGTAACTATGACACGTCTTGGGTTCCGCCAGACTCCAGAGAA
GAGACTGGAGTGGGTACATCTATTAGTAGTGGTGGTTACACCTACTATCCAGACAGTGCGAAG
GGCCGATTCAACATCTCCAGAGATAATGCCAGGAACATCCTGTACCTGCAAATGAGCAGTCTGA
GGTCTGAAGACACGGCCATGTATTACTGTGCAAGAGCCCTTTATGACTACACTACTAGCCCCTG
GTTTGCTTACTGGGGCCAAGGGACTCTGGTCATGTCTCTC**GCCAAAACGACACCCCCATCTGTC**
TATCCACTGGCCCCCTGGATCTGCTGCCCAAATAACTCCATGGTGACCCTGGGATGCCTGGTCA
AGGGCTATTTCCCTGAGCCAGTGACAGTGACCTGGAACCTCTGGATCCCTGTCCAGCGGTGTGCA
CACCTTCCCAGCTGTCTGAGTCTGACCTCTACACTCTGAGCAGCTCAGTGACTGTCCCCTCC
AGCACCTGGCCCAGCCAGACCGTCACCTGCAACGTTGCCACCCGGCCAGCAGACCAAGGTGG
ACAAGAAAATTGTGCCCAGGGATTGTGGC

5'3' Frame 1

EVQLQQSGGGLVKPGGSLKLSAASGFTFSNYDTSWVRQTPEKRLEWVTSISSGGYTYYPDSAK
GRFTISRDNARNILYLQMSSLRSEDAMYYCARALDYTTSPWFAYWGQGLVMSL**AKTTPPSV**
YPLAPGSAAQTNSMVTLGCLVKGYFPEPVTVTWNSGSLSSGVHTFPAVLQSDLYTLSSSVTVPS
STWPSQFTVCNVAHPASSTKVDKKIVPRDCG

(b) 80.2 LC:

GACATTGTGCTGACCCAATCTCCAGCAATCATGTCTGCATCTCCAGGGGAAAAGGTCACCATGA
CCTGCAGGGCCAGCTCAAGTGTAAGTTCCAGTTACCTGCACTGGTACCAGCAGAAGTCAGGTGC
CTCCCCCAAATCTGGATTTATAGCACATCCAACCTGGCCTCTGGAGTCCCTGCTCGCTTCAGT
GGCAGTGGGTCTGGGACCTCTTACTCTCTCACAATCAGCAGTGTGGAGGCTGAAGATGCCGCCA
CTTATTACTGCCAGCAGTACAGTGGTACCCCGCTCACGTTCTGGTGGTGGGACCAAGCTGGAGCG
AAAC**GCTGATGCTGCACCAACTGTATCCATCTTCCCACCATCCAGTGAGCAGTTAACATCTGGA**
GGTGCCTCAGTCGTGTGCTTCTTGAACAACCTTCTACCCCAAAGACATCAATGTCAAGTGGAAGA
TTGATGGCAGTGAACGACAAAATGGCGTCCTGAACAGTTGGACTGATCAGGACAGCAAAGACAG
CACCTACAGCATGAGCAGCACCCCTCACGTTGACCAAGGACGAGTATGAACGACATAACAGCTAT
ACCTGTGAGGCCACTCACAAGACATCAACTTCACCCATTGTCAAGAGCTTCAACAGGAATGAGT
GTGGC

5'3' Frame 1:

DIVLTQSPAISASPGEKVTMTCRASSSVSSSYLHWYQQKSGASPKLWIYSTSNLASGVPARFS
GSGSGTSYSLTISSVEAEDAATYYCQQYSGTPLTFGAGTKLERN**ADAAPTVSIFPPSSEQLTSG**
GASVVCFLNNFYPKDINVWKIDGSERQNGVLNSWTDQDSKSTYSMSSTLTTLTKDEYERHNSY
TCEATHKTSTSPIVKSFNRECG

Figure 4.

Nucleotide and corresponding amino acid sequences of (a) 80.2 HC from V_H and C_H1 and
(b) 80.2 LC from V_L and C_L. Variable regions are shown in regular font, constant regions in
bold.

Figure 5. SDS-PAGE analysis of the refolded 80.2 Fab, purified by IMAC. Lane 1, eluate Fab; lane 2, eluate Fab; lane 3, Fab under reducing conditions.

Figure 6.

Dot blot analysis of the 80.2 Fab upon refolding. Dot 1, non-refolded Fab; dot 2, blank, no antibody protein; dot 3, BL21 (DE3) non-transformed cells; dot 4, Fab after refolding; dot 5, blank; dot 6, 80.2 parent antibody as positive control. 2 μ g protein was blotted for all samples.

Figure 7.

Assessment of the stereoselectivity of refolded 80.2 Fab as determined by non-competitive ELISA. *p*-Azo-L-Phe-BSA (), *p*-azo-D-Phe-BSA () and BSA (), respectively, were used as solid phase coatings.

Figure 8. Results obtained in competitive ELISAs performed with 80.2 Fab antibody using the L-enantiomers (□) or D-enantiomers (■) of (a) phenylalanine, (b) p-amino phenylalanine, (c) tryptophan, and (d) cyclohexylalanine as competitors. Error bars represent standard deviations of triplicate determinations.

Table 1

Effect of the induction time on protein expression yields.

Protein	Time of induction (h)	Yield (mg/1 L culture)
80.2 HC	0	50
80.2 LC	0	57
80.2 HC	4	178
80.2 LC	4	166
80.2 HC	8	130
80.2 LC	8	127
80.2 HC	16	100
80.2 LC	16	100

Table 2

Relative affinities of Fab 80.2 for various L-amino acids as determined by competitive ELISA.

-amino acid	Structure	IC ₅₀
L-Phenylalanine		2.55mM
<i>p</i> -Amino-L-phenylalanine		5.04 mM
L-Tryptophan		15.5 mM
L-Cyclohexylalanine		50.1 mM