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Single-step purification of a bacterially expressed antibody F_v fragment by immobilized metal affinity chromatography in the presence of betaine

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ABSTRACT

A procedure was developed for the rapid isolation of an antibody F_v fragment expressed in *Escherichia coli* via immobilized metal affinity chromatography. Metal affinity was mediated by fusing hexahistidine tails to both the V_L and the V_H domain and was thus independent of the antigen-binding specificity. Unexpectedly, it was not possible to isolate the F_V fragment with correct stoichiometric composition of the two variable domains under standard chromatographic conditions. Proper non-covalent association of V_L and V_H was, however, maintained when using glycine betaine as electrolyte, thus permitting purification of the intact F_v fragment to homogeneity in a single step.

INTRODUCTION

Since the development of methods for the expression of functional F_v and F_{ab} fragments of antibodies in *Escherichia coli* [1–3] the bacterial production of recombinant immunoglobulin fragments has gained widespread application (for a review see ref. 4). Initially hapten or antigen affinity columns were used for the purification of functional antibody fragments from the periplasmic fraction, whole-cell extract or culture supernatant of *E. coli*. Recently, methods have been developed that permit the affinity purification of bacterially expressed Ig fragments independent of their antigen-binding properties [5,6]. These methods, which rely on C-terminally fused peptide sequences conferring specific affinity properties, are particularly advantageous in cases where antigens are too valuable, unstable or just unavailable for the preparation of affinity matrices.

A sequence of five or six consecutive histidine residues fused to a recombinant protein has been shown to enable specific purification of the polypeptide via immobilized metal affinity chromatography (IMAC) [7,8]. This approach was also successfully employed for the facile purification of an active single-chain F_v fragment produced in *E. coli* [5]. The Ig fragment retained its original antigen-binding properties so that the carboxy-terminal oligohistidine tag did not need to be removed. In contrast, attempts to purify an F_v fragment without a peptide linker connecting the two variable domains using the same strategy did not lead to immediate success, because of

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chain dissociation. We demonstrate here that the non-covalent association of these two polypeptide chains, can, however, be sufficiently stabilized by use of a buffer system containing the osmolyte glycine betaine instead of commonly used inorganic salts, thus enabling IMAC purification of the intact F_v fragment from *E. coli* in a single step.

EXPERIMENTAL

Chemicals

Analytical grade chemicals were purchased from Merck (Darmstadt, Germany) chelating Sepharose fast flow was from Pharmacia (Freiburg, Germany), glycine betaine (monohydrate) and tetramethylammonium chloride from Fluka (Neu-Ulm, Germany), Coomassie brilliant blue R-250 from Serva (Heidelberg, Germany), ampicillin from Sigma (Deisenhofen, Germany) and isopropyl β-D-thiogalactopyranoside (IPTG) from Biomol (Hamburg, Germany).

Bacterial expression of the antibody F_v fragment

The expression plasmid pASK69-M29b was constructed from the vector pASK30 described previously [5] by standard DNA manipulations [9]. Unique restriction sites were introduced at the beginning and the end of the V_H and V_L genes in order to permit the cloning and expression of a variety of different variable domain genes, obtained, for example, by polymerase chain reaction [10]. pASK69-M29b was used for expression of the mutant variant "M29b" of the F_v fragment derived from the anti-lysozyme antibody HyHEL-10 [11]. The coding regions for the variable domains of this Ig fragment were obtained by gene synthesis, as will be described in detail elsewhere [12].

A 2-l culture of *Escherichia coli* strain JM83 [*ara*, Δ(*lac-pro*AB), *rps*L (=*str*A), φ80, *lac*-ZΔM15] [13] transformed with the expression plasmid was grown at 22°C in Luria-Bertani (LB) medium [9] containing 100 μg/ml ampicillin. Expression was induced at an A_{550} value of *ca.* 0.5 by the addition of 0.5 m*M* IPTG (final concentration). After an induction period of 3 h the bacterial cells were harvested by centrifugation, resuspended in 20 ml of ice-cold 0.5 *M* NaCl, 50 m*M* sodium phosphate, pH 7.5, 1 m*M* EDTA and stirred for 30 min at 4°C. The spheroplasts were sedimented by centrifugation at 4400 *g* for 30 min at 4°C. The supernatant was recovered as the periplasmic cell fraction and cleared by centrifugation at 27 000 *g* for 15 min at 4°C.

IMAC

A Zn^{2+}/IDA (iminodiacetic acid) Sepharose matrix was prepared by charging a column packed with 2 ml (bed volume) of chelating Sepharose fast flow with 15 ml of 10 m*M* $ZnSO_4$ followed by washing with water. The column was equilibrated with one of the following chromatography buffers (all steps were carried out at 4°C): (i) 1 *M* NaCl, 40 m*M* sodium phosphate, pH 7.0; (ii) 0.5 *M* K_2SO_4, 50 m*M* sodium phosphate, pH 7.0; (iii) 0.5 *M* glycine betaine, 50 m*M* sodium phosphate, pH 7.0. The periplasmic cell fraction from a 2-l *E. coli* culture (*ca.* 20 ml, see above) dialysed against the same buffer was applied to the column, which was subsequently washed with approximately ten bed volumes of the chromatography buffer until absorption of the flow-through reached the baseline. Then bound protein was eluted with a linear gradient of 0 m*M* to 500 m*M* imidazole in the chromatography buffer (during this step the flow-rate was reduced from 20 ml/h to 10 ml/h).

Sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE of column fractions was performed using the discontinuous buffer system according to Fling and Gregerson [14] followed by staining with Coomassie brilliant blue. For quantitative determination of V_H/V_L ratios the gel lanes were scanned at 585 nm on a GS300 densitometer (Hoefer Scientific Instruments, San Francisco, CA, USA) connected to an IBM personal computer using analysis software from the same manufacturer.

RESULTS AND DISCUSSION

The experiments described were aimed at the purification of a genetically engineered F_v frag-

ment with a modified amino acid sequence [12] based on the primary structure of the antibody HyHEL-10 [11]. For bacterial production of the F_v fragment in a functional state the two variable domains, V_H and V_L, which are non-covalently associated, were co-secreted into the periplasm of the same *E. coli* cell. In this compartment disulphide bond formation, protein folding and chain association were shown to take place [1]. For this purpose a suitable expression vector, pASK69-M29b, was constructed (Fig. 1). On this plasmid, V_H and V_L were both fused at their N-termini to bacterial signal sequences directing transport across the inner cell membrane, and their structural genes were arranged in an artificial dicistronic operon in order to permit inducible coexpression.

For purification of the recombinant F_v fragment via IMAC, initially a single oligohistidine tail (five or six residues) was fused to the C-terminus of either V_H or V_L. Yet, in preliminary experiments the F_v fragment could not be isolated in either of these cases. Instead, only that variable domain bound to the Zn^{2+}/IDA Sepharose column which carried the C-terminal affinity tag (not shown) and was then individually eluted by the imidazole gradient under standard chromatographic conditions [5].

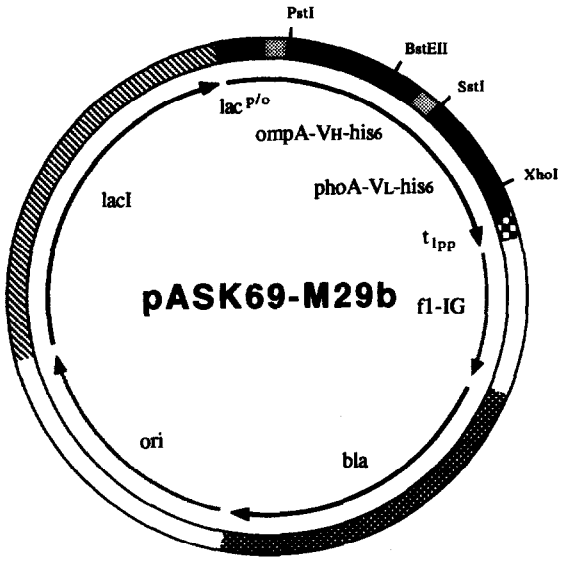

Fig. 1. Schematic drawing of the plasmid pASK69-M29b for the production of the antibody F_v fragment in *E. coli*. The variable domain genes V_H and V_L are bounded by singular restriction sites as indicated. They were both fused at their 5′ ends to nucleotide sequences encoding bacterial signal peptides from the outer membrane protein A (*ompA*) and from alkaline phosphatase (*phoA*), respectively, and at their 3′ ends to six consecutive histidine codons. The two structural genes were arranged in an artificial dicistronic operon under control of the IPTG-inducible *lac* promoter. *lpp*, f1-IG, *bla* and *ori* denote the strong lipoprotein transcription terminator, the intergenic region from the single-stranded bacteriophage f1, the β-lactamase gene for ampicillin resistance and the origin of replication, respectively.

Consequently it was sought to confer similar metal affinities on both polypeptide chains by adding hexahistidine tails to the C-termini of V_H as well as V_L. When the periplasmic cell fraction of *E. coli* cells harbouring the corresponding expression plasmid pASK69-M29b (see Fig. 1) was applied to a Zn^{2+}/IDA Sepharose column, the proteins that bound to the metal chelate matrix were eluted in two peaks by the imidazole gradient (Fig. 2a). The early peak at low imidazole concentrations contained host cell proteins with low affinity for immobilized metal ions (not shown), whereas the two variable domains of the F_v fragment were together specifically eluted in a single symmetric peak at higher eluent concentrations. Unexpectedly, however, an analysis of individual protein fractions by SDS-PAGE revealed a non-stoichiometric ratio for the two polypeptide chains with a large excess of V_L over V_H (Fig. 2b and c).

It was already known that immunoglobulin V_L domains can adopt soluble globular structures by homodimerization to form so-called Bence Jones proteins [15]. A V_L dimer has similar size and shape as an F_v fragment, i.e. the heterodimer of V_H and V_L. Thus, it had to be concluded that a mixture of the F_v fragment and its corresponding V_L dimer was isolated by the chromatographic procedure employed, and the question arose whether it was possible to separate these two protein species by the choice of appropriate buffer conditions.

IMAC of native proteins is generally performed in the presence of high-salt conditions in order to prevent non-specific binding to the charged groups of the affinity matrix via an ion-exchange effect. Ordinarily, NaCl is used for this purpose, but the use of K_2SO_4 has also been reported [16]. Yet, when the same chromatog-

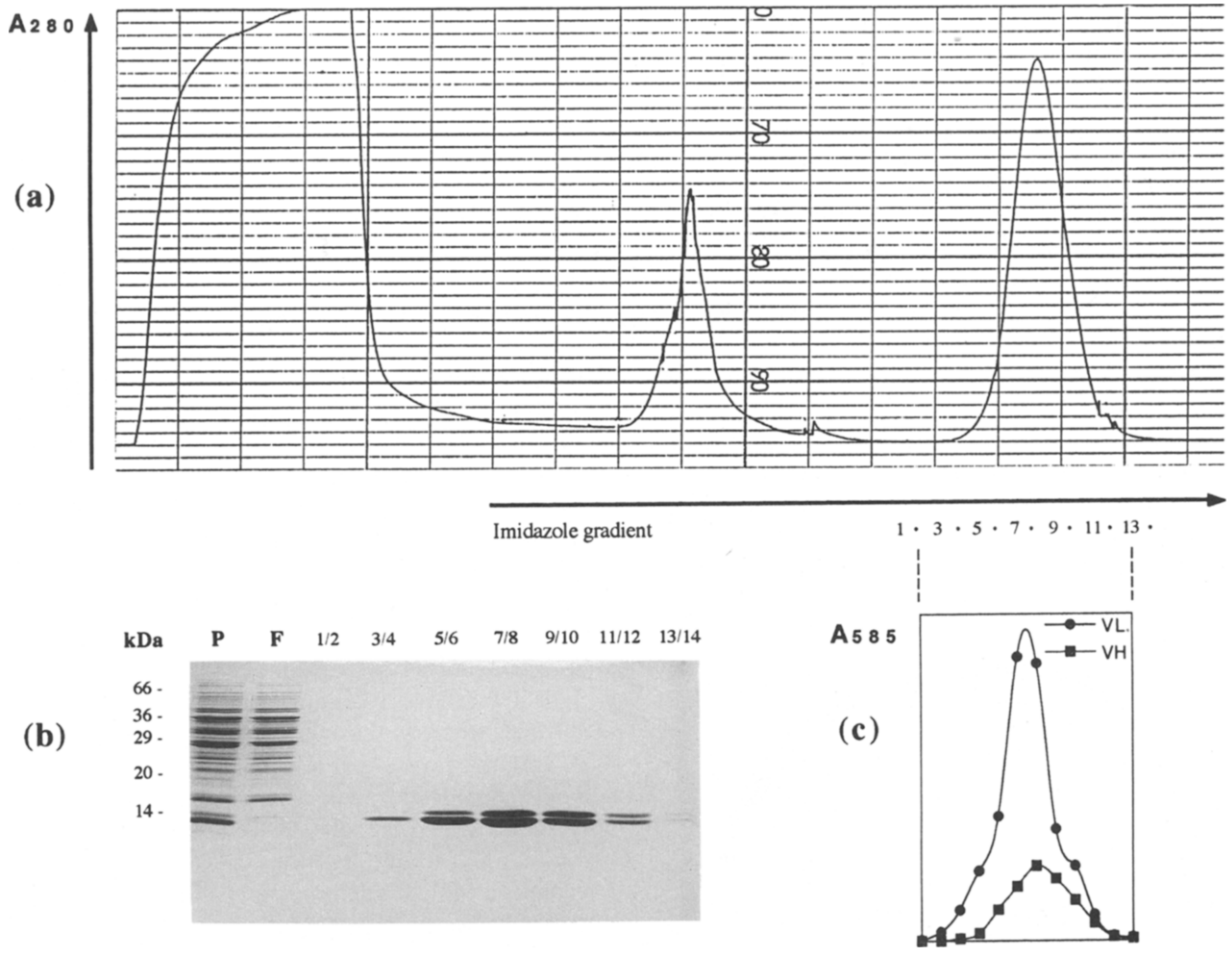

Fig. 2. IMAC in the presence of 1 *M* NaCl of the bacterial periplasmic cell fraction containing recombinant F_v fragment. (a) Elution profile monitored at 280 nm. (b) SDS-PAGE (18%) showing consecutive peak fractions (pooled in pairs) as indicated below (a). Lane P, periplasmic cell fraction; lane F, flow-through. Molecular masses are given to the left in kilodaltons (kDa). V_L corresponds to the lower and V_H to the upper of the two bands in the peak fractions. (c) Elution profiles of the variable domains V_H and V_L separately determined by scanning of peak fractions on an SDS-polyacrylamide gel (absorption in arbitrary units).

raphy experiment was carried out as before with 0.5 *M* K_2SO_4 as electrolyte instead of 1 *M* NaCl, again a single symmetrical elution peak was obtained (Fig. 3a) representing a non-stoichiometric mixture of V_L and V_H (Fig. 3b and c). This time, however, elution occurred at significantly higher imidazole concentrations, and it appeared unlikely that the F_v fragment and the V_L dimer displayed identical metal affinity properties in the presence of both inorganic salts.

Rather, we had to consider the possibility of a dynamic equilibrium between both species that was favoured by the high ionic strength conditions, effectively preventing their separation during chromatography. Thus, in the third experiment an electrolyte was chosen that did not just primarily have an effect on the ionic strength of the buffer but that also potentially contributed to the stabilization of the non-covalent association of the variable immunoglobulin domains. Recently, it has been reported that the osmolyte glycine betaine exerts a stabilizing effect on proteins against thermal denaturation [17]. Glycine betaine is a highly soluble zwitterionic compound that combines a negatively charged carboxylate group and a positively charged

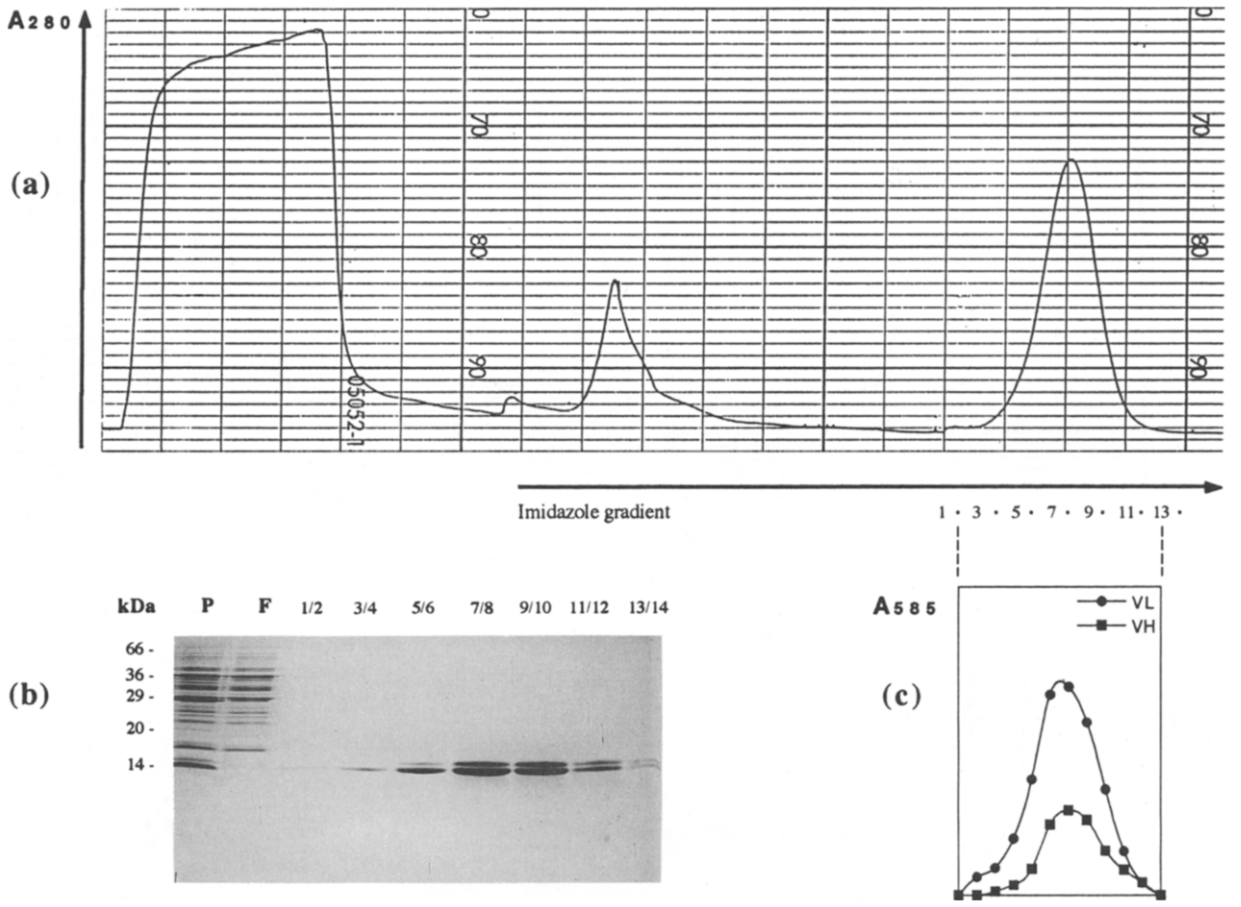

Fig. 3. IMAC in the presence of 0.5 *M* K_2SO_4 of the bacterial periplasmic cell fraction containing recombinant F_v fragment. (a), (b) and (c) as described for Fig. 2.

quaternary ammonium group in the same molecule and thus strongly contributes to the ionic strength of the solution. Since the nitrogen is permethylated in betaine it cannot act as a ligand for metal ions via a free electron pair and was thus not expected to interfere with metal complexation.

When IMAC was carried out in the presence of 0.5 *M* betaine a similar amount of protein bound to the column as before but, remarkably, the recombinant protein was eluted in two distinct peaks by the imidazole gradient under otherwise unchanged conditions (Fig. 4a). SDS-PAGE revealed that the second peak obtained corresponded to the pure F_v fragment with stoichiometric composition of V_H and V_L, whereas the first peak represented the excess amount of the V_L domain (Fig. 4b and c). Upon rechromatography, the isolated F_v fragment appeared to be homogeneous and stable (not shown). Thus, the use of betaine as electrolyte enabled the efficient purification of the intact F_v fragment from the periplasmic cell fraction of *E. coli* in a single step. In order to clarify the precise role of glycine betaine in the observed effect, another IMAC experiment was performed in the presence of 0.5 *M* tetramethylammonium chloride. As a result, exactly the same elution profile was obtained (not shown). Therefore, it has to be concluded that the stabilizing effect of betaine during IMAC is mainly due to the hydrophobic nature of the tetraalkylammonium ion.

Meanwhile, IMAC in the presence of betaine

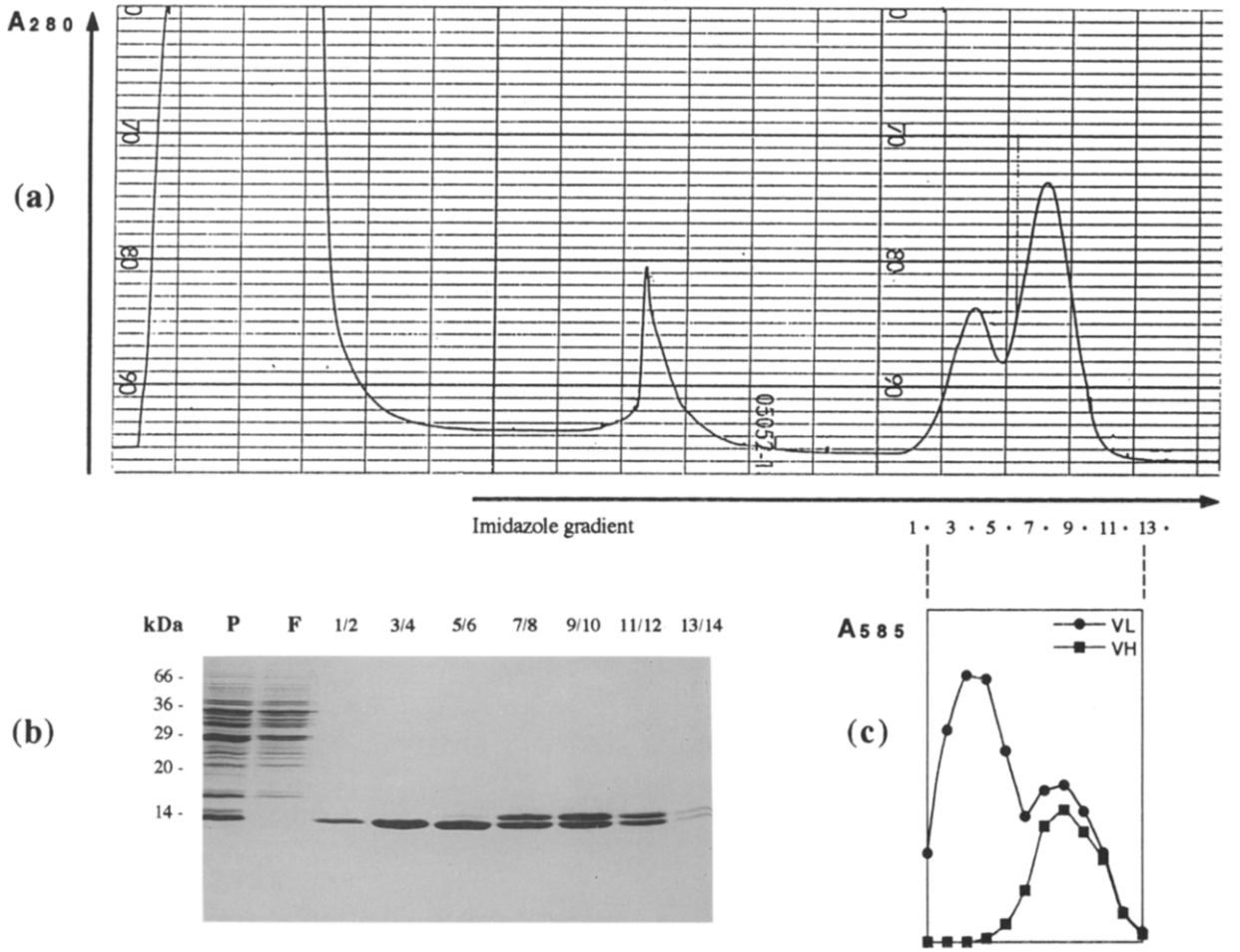

Fig. 4. IMAC in the presence of 0.5 *M* glycine betaine of the bacterial periplasmic cell fraction containing recombinant F_v fragment. (a), (b) and (c) as described for Fig. 2.

was used for the successful isolation of several different F_v fragments in our laboratory, indicating that the chromatographic conditions established here generally serve to stabilize the non-covalent association between the variable immunoglobulin domains. It should be noted that in the purification of a single-chain F_v fragment via IMAC described before [5] the variable domains were covalently connected by a flexible peptide linker so that no special care had to be taken to ensure the integrity of chain dimerization. Nevertheless, the purification of a functional and homogeneous antibody F_v fragment with proper chain stoichiometry had been possible before in a physiological buffer system by fusing a peptide conferring binding affinity towards immobilized streptavidin to the C-terminus of just one of the variable domains [6]. Thus, the dynamic behaviour observed here for the chain association of V_H and V_L could not be expected *a priori* and has to be attributed to the high ionic strength conditions employed for IMAC. The results presented clearly demonstrate the important role of the buffer conditions in the purification of an intact antibody F_v fragment, particularly if the antigen or hapten is absent. The stabilizing effect of betaine and related compounds may be of relevance not only for the isolation of this particular Ig fragment but also for the purification of protein complexes composed of several subunits via IMAC in general.

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