Journal of Peptide Science
J. Peptide Sci. **10**: 350–362 (2004)
Published online 21 January 2004 in Wiley InterScience (www.interscience.wiley.com). DOI: 10.1002/psc.550

JOURNAL OF PEPTIDE SCIENCE

Peptides and Multiple Antigen Peptides from *Schistosoma mansoni* Glyceraldehyde 3-Phosphate Dehydrogenase: Preparation, Immunogenicity and Immunoprotective Capacity in C57BL/6 Mice‡

PAVEL VEPŘEK,[a] JAN JEŽEK,[a]* JIŘÍ VELEK,[a] HATEM TALLIMA,[b] MONA MONTASH[b] and RASHIKA EL RIDI[b]

[a] Institute of Organic Chemistry and Biochemistry, Academy of Sciences of the Czech Republic, Flemingovo nám. 2, 166 10 Prague 6, Czech Republic
[b] Department of Zoology, Faculty of Sciences, Cairo University, Cairo 12613, Egypt

Received 16 July 2002
Accepted 15 October 2003

Abstract: Four monoepitopic MAPs (MAP A, B, C and E) and one bis-diepitopic MAP B-E derived from the primary sequence of *Schistosoma mansoni* glyceraldehyde 3-phosphate dehydrogenase, previously tested in BALB/c mice, were examined for their immunogenicity and protective capacity in C57BL/6 mice. Despite multimerization into MAPs, MAP A and MAP C were poorly immunogenic. In contrast to BALB/c mice, MAP E was non-immunogenic in C57BL/6 mice. Peptide B in the form of MAP B or bis-diepitopic MAP B-E elicited immune responses in C57BL/6 mice that were associated with a significant decrease in worm burden.

The MAPs were prepared by the stepwise solid-phase peptide synthesis using Boc/Bzl chemistry, successfully purified on the RP-HPLC column and characterized by RP-HPLC, HPCE and MALDI-TOF MS techniques. A general strategy for MAPs purification is discussed here and the purification of MAP B and MAP E is documented in detail.

Keywords: multiple antigen peptides (MAPs); *Schistosoma mansoni*; peptide vaccine; SG3PDH; RP-HPLC purification

INTRODUCTION

There are a number of species of schistosomes that can infect humans. However, most of the human infections are caused by *S. mansoni*, *S. haematobium* and *S. japonicum*. *S. mansoni* infects approximately 200 million people in 74 countries resulting in 1 million deaths annually [3]. The infection is initiated by the penetration of infective cercariae through the human skin, followed by its transformation into a schistosomula that migrates into lungs and then to the liver where the worm matures, copulates and begins the deposition of eggs. A proportion of the eggs make their way to the exterior with stools, whereas the rest are trapped in the host tissues leading to severe inflammation, granuloma formation and ultimately

Abbreviations: Standard abbreviations have been followed throughout this paper (*J. Peptide Sci.* 2003; **9**: 1–8). CHCA, alpha-cyano-4-hydroxycinnamic acid; Con A, concanavalin A; G3PDH, glyceraldehyde 3-phosphate dehydrogenase; HPCE, high-performance capillary electrophoresis; IFN-γ, interferon-gamma; IL, interleukin; MeCN, acetonitrile; rSG3PDH, recombinant *S. mansoni* glyceraldehyde 3-phosphate dehydrogenase; SG3PDH, *S. mansoni* glyceraldehyde 3-phosphate dehydrogenase.

* Correspondence to: Jan Ježek, Institute of Organic Chemistry and Biochemistry, Academy of Sciences of the Czech Republic, Flemingovo nám. 2, 166 10 Prague 6 — Dejvice, Czech Republic; e-mail: jezek@uochb.cas.cz

‡ Part of this work was presented in a preliminary form on Symposia, see refs [1,2].

Contract/grant sponsor: CEZ:AV0Z4055905, Grant Agency of the Czech Republic; Contract/grant number: 303/01/0690.
Contract/grant sponsor: ICGEB; Contract/grant number: CRP/EGY98-03.

fibrosis. Control of the disease is now accomplished by praziquantel chemotherapy, but the problem of frequent reinfection stresses the need for a safe, stable and effective vaccine [4].

Schistosoma mansoni glyceraldehyde 3-P dehydrogenase (SG3PDH) is a target of cellular and humoral immune responses of Brazilian and Egyptian subjects putatively resistant to reinfection with *S. mansoni* [4–9]. Like most schistosoma vaccine proteins, the amino acid sequence of SG3PDH shows numerous positional identities with its mammalian counterpart [6]. Vaccination with the whole parasite protein or conserved regions of the molecule may potentially induce autoimmune responses. Therefore, it is important to select the least conserved polypeptides of the molecules to use as vaccines. It is also important to consider the problem that these peptides must induce optimal T and B cell activities. Peptides fulfilling such criteria have been selected from the sequences of SG3PDH [10], glutathione S-transferase [11], Sm23 [12] and triose phosphate isomerase [13]. Previous studies by Argiro *et al.* [10] attempted to define T and B cell determinants in SG3PDH, and assessed whether peptides corresponding to the antigenic epitopes can be used in a subunit vaccine. Our approach was different, as peptides derived from its primary sequence were selected based on the lowest homology to the human G3PDH. The selected peptides were shown to carry human and murine T and B cell determinants [14].

Synthetic peptides are generally poor immunogens and therefore they are usually conjugated to protein carriers to produce effective antibodies. However, (i) ambiguous chemical composition and structure, (ii) often immunodominancy of a protein carrier, and (iii) low ratio of an antigen to a carrier are major drawbacks of their use. MAPs (multiple antigen peptides) are an efficient alternative to peptide–protein conjugates [15]. They lack any of the above-mentioned disadvantages and therefore they possess great potential as immunogens or synthetic vaccines [16]. Therefore, the MAP pattern of SG3PDH-derived peptides was used to investigate the immunogenicity and protective efficacy in BALC/c [17] and C57BL/6 mice.

The purification of MAPs is a very challenging task even for experienced peptide chemists. Rare examples dealing mainly with the purification of ligation products [18] have been published. Basak *et al.* [19] published the preparation and purification of an 8-branched MAP, however, no RP-HPLC or HPCE spectra were included. ES-MS spectra were not very clear and multiply charged ion peaks were unacceptably broad. This is, of course, due to their branched character, which gives the molecules their unique physicochemical characteristics that have an unfortunate deteriorating effect on separation compared with their linear analogues. Attempts to purify MAPs have focused mainly on the partial fractionation by gel permeation chromatography (GPC) or dialysis. None the less, the purification of MAPs via RP-HPLC is still the method of choice. In this paper, the strategy used for their successful purification is discussed, documented by RP-HPLC, HPCE and MALDI-TOF spectra.

MATERIALS AND METHODS

Acetonitrile, dichloromethane, *N*,*N*′-dimethylformamide and tert-butyl methyl ether were purchased from Fluka (Buchs, Switzerland). Boc-amino acids were obtained from Bachem (Bubendorf, Switzerland). The protected amino acids used were Boc-Arg(Tos), Boc-Asp(OcHx), Boc-Cys(MeBzl), Boc-Glu(OcHx), Boc-His(Dnp), Boc-Lys(Boc), Boc-Lys(Fmoc), Boc-Lys(2-ClZ), Boc-Ser(Bzl), Boc-Thr(Bzl), Boc-Trp(For) and Boc-Tyr(2-BrZ). 4-methyl benzhydrylamine resin (0.57 mmol NH_2/g), Boc-Val-PAM, Boc-Asp(OcHx)-PAM, Boc-Ala-PAM resins (0.5–0.6 mmol Boc-AA/g) were purchased from Calbiochem-Novabiochem (Laufelfingen, Switzerland).

Peptide Synthesis

Peptides A, B, C and E were synthesized manually on the corresponding pre-loaded PAM resins. MAPs A, B, C and E were synthesized on the [Boc-Lys(Boc)]-Lys[Boc-Lys(Boc)]-Gly-NH-MBHA resin with initial substitution reduced to approx. 0.175 mmol/g. Both peptide acids and MAP amides were synthesized by Boc/Bzl strategy. Couplings were performed in 3-fold molar excess of amino acid using DCC/HOBt or BOP/DIEA activation. The coupling efficacy was monitored by ninhydrin assay [20]. If necessary, DMAP was added and the coupling time was extended by 2 h. If the condensation was still incomplete, the coupling was repeated using BOP/DIEA activation. The bis-diepitopic MAP B-E was prepared in an analogous way using *in situ* neutralization [21] and the activation by HBTU/DIEA. *N*-terminal amino groups of MAP B-E were acetylated in Ac_2O/pyridine/DMF (1 : 2 : 10, v/v/v, 10 fold molar excess) for 30 min. Deprotection of His(Dnp) and Trp(For) was achieved as follows: Dnp was

split off by 5% thiophenol in DMF (5 × 1 h) followed by a thorough DMF wash (5 × 1 min). The For group was removed by treatment with 10% piperidine in DMF (1 h at 0 °C), followed by a DMF wash (5 × 1 min). After the removal of the final Boc-group, the peptide-resin was thoroughly washed with DMF, DCM, ethanol and ether and dried under vacuum. Peptides and MAPs were deprotected and detached from the resin by treatment with HF/anisole (9 : 1) for 1 h [22]. The scavengers were washed out by ether (for peptides containing Met tert-butyl methyl ether was used), the crude product was extracted with 15% AcOH and freeze-dried. Crude products were analysed and purified by RP-HPLC. The yields of peptides and MAPs were calculated in relation to the first amino acid on the resin and are not corrected for solvation. The list of prepared peptides and MAPs together with their analytical data is given in Table 1.

Preparation of the (Boc-Lys(Boc))-Lys(Boc-Lys(Boc))-Gly-NH-resin

MBHA resin, 0.57 mmol NH_2/g (3.5 g; 2 mmol), was solvated in DMF for 20 min and then neutralized by 5% DIEA in DMF (v/v). Boc-Gly-OH (2 mmol) was coupled for 30 min using DCC/HOBt activation. Quantitative AAA afforded substitution 0.175 mmol Gly/g of the resin (the average value of three hydrolyses). Unreacted amino groups were acetylated. Two levels of Lys were built up. The peptide resin was then washed with DMF, DCM and ether and then dried in vacuum. The yield was 4.84 g of [Boc-Lys(Boc)]-Lys[Boc-Lys(Boc)]-Gly-NH-resin. AAA (20 h hydrolysis): Lys 2.89 (3), Gly 1.0 (1).

General Procedures

High-performance liquid chromatography. Peptides and MAPs were analysed on a LaChrom (Merck) HPLC machine equipped with an L-7100 pump, L-7450 diode array, D-7000 interface and LiChrospher WP300 C_{18} (250 × 4.0 mm i.d., 5 μm) or Vydac Protein C_4 214TP54 (250 × 4.6 mm i.d., 5 μm) column using D-7000 HSM ver. 4.0 software. The gradients are detailed further in the Results section. The peptides and MAPs were purified on the preparative RP HPLC apparatus (Beckman, USA) equipped with 114M pump, 421 controller and SPD-2A detector (Shimadzu, Japan). The columns used were Vydac Peptide and Protein C_{18} 218TP10220 (250 × 22.5 mm i.d., 10 μm) for peptides and Vydac Protein C_4 214TP1010, (250 × 10 mm i.d., 10 μm) for MAPs and with water–acetonitrile gradients (both phases contained 0.1% TFA or AcOH v/v).

High-performance capillary electrophoresis. Analyses were performed in a homemade apparatus for high-performance capillary electrophoresis (HPCE) [23] equipped with untreated fused silica capillary (i.d. 0.05 mm, o.d. 0.200 mm, effective length 190 mm, total length 300 mm, polyimide outer coating) and UV photometric detector at 206 nm. The peptides were dissolved in the background electrolyte (BGE) or in water in the concentration range 0.5–0.75 mg/ml. Peptides and MAPs were analysed in the mode of capillary zone electrophoresis (CZE) as cations in acidic BGE I (100 mM H_3PO_4, 50 mM Tris, pH 2.25) or as anions in weakly alkaline BGE II (40 mM Tris, 40 mM tricine, pH 8.1). A nanolitre sample volume was introduced into the capillary hydrodynamically with pneumatically induced overpressure (500–700 Pa) for 10–20 s. The applied voltage was 10.0 kV (anode at the injection end of the capillary), electric current was 56.0 μA in BGE I and 8 μA in BGE II at ambient temperature 23 °C.

Mass spectrometry. Positive-ion FAB spectra were recorded on a ZAB-EQ mass spectrometer (VG Analytical, Manchester, UK), with an IonTech gun and glycerol or glycerol + thioglycerol matrix. Positive MALDI-TOF spectra were recorded on a Reflex IV (Bruker Daltonics, USA) in reflectron mode (HV 20 kV). The matrix used was CHCA (alpha-cyano-4-hydroxycinnamic acid) dissolved in 0.1% TFA and MeCN. Samples were dissolved in water +0.1% TFA into a final concentration of 1 mg/ml.

Amino acid analysis. The amino acid composition of both peptides and MAPs was determined on a Biochrom-20 Pharmacia Amino Acid Analyzer (Pharmacia LKB Biochrom Ltd, Cambridge, England). Prior to analysis, the samples were hydrolysed in 6N HCl at 110 °C for 20 or 72 h.

Immunology

Mice and parasites. Eight to 10-week-old female C57BL/6 mice were raised and maintained throughout the experimentation in the Schistosome Biological Supply Program, Theodore Bilharz Research Institute, Cairo, Egypt (SBSP). Cercariae and adult worms of an Egyptian strain of *S. mansoni* were obtained from SBSP.

Table 1 Prepared Peptides and MAPs and their Analytical Data

Compound	Sequence		[M + H]$^+$		Yield [%]	Amino acid analysis found															
			Calc.	Found		K	H	R	D/N	T	S	E	P	G	A	V	M	I	L	Y	W
Peptide A	LKNTVDVVSV-OH (AA 24–33)	$C_{47}H_{84}N_{12}O_{16}$	1073.2	1073.3	38.0	0.95			1.96	0.92	0.91					4.0			0.93		
Peptide B	ERDPANIPWDKD-OH (AA 78–89)	$C_{63}H_{94}N_{18}O_{22}$	1455.6	1455.8	28.5	1.0		1.03	3.88			1.05	1.93		1.02			0.97			0.92
Peptide C	ENSYEKSMSVV-OH (AA 138–148)	$C_{53}H_{85}N_{13}O_{21}S$	1272.4	1271.8	23.8	1.02			1.0		2.85	2.11				2.0	0.90			0.92	
Peptide E	ITHMHKVDHA-OH (AA 329–338)	$C_{51}H_{81}N_{17}O_{14}S$	1188.4	1188.5	17.3	0.98	2.72		0.96	0.92					1.0	1.05	0.91	1.03			
MAP A	(LKNTVDVVSV)$_4$K$_2$KG-NH$_2$	$C_{208}H_{370}N_{56}O_{64}$	4679.6	4676.7	22.9	6.92			8.0	3.41	3.25			1.13		14.16			3.93		
MAP B	(ERDPANIPWDKD)$_4$K$_2$KG-NH$_2$	$C_{272}H_{410}N_{80}O_{88}$	6208.8	6205.0	18.2	7.26		3.87	16.3			4.0	7.8	1.1	3.96			3.97			3.05
MAP C	(ENSYEKSMSVV)$_4$K$_2$KG-NH$_2$	$C_{232}H_{374}N_{60}O_{84}S$	5476.2	5474.2	31.1	6.67			3.99		7.45	7.2		1.1		8.0	3.86			3.76	
MAP E	(ITHMHKVDHA)$_4$K$_2$KG-NH$_2$	$C_{224}H_{358}N_{76}O_{56}S_4$	5140.1	5140.7	58.2	6.6	10.9		3.86	3.31				1.09	4.0	3.81	3.58	3.65			
MAP B-E	[Ac-ERDPANIPWDKDK (Ac-ITHMHKVDHA)]$_2$KG-NH$_2$	$C_{256}H_{394}N_{78}O_{76}S_2$	5844.5	5844.9	25.0	9.22	6.6	2.1	9.07	1.81		2.04	4.26	1.1	4.07	1.93	1.94	4.0			1.92

[a] Acid hydrolysis (72 h, 110 °C, 6N HCl).
[b] Acid hydrolysis (20 h, 110 °C, 6N HCl).

Antigens. The coding sequence for SG3PDH was obtained and expressed, and the recombinant protein rSG3PDH was purified by metal affinity chromatography (HiTrap™ Affinity Column, Pharmacia, Uppsala, Sweden) as detailed previously [14].

Immunization and challenge. In the first experiment, 50 C57BL/6 mice were assigned randomly to five groups, 10 mice each. Group 1 was injected with adjuvant and considered as a negative control. The other groups were immunized with an appropriate MAP construct (50 μg of MAP per mouse per injection).

In the second experiment, 30 mice were assigned randomly to three groups, 10 mice each. The mice of the first group were injected with adjuvant and considered as negative controls. The mice of the second group were immunized with 50 μg per mouse of bis-diepitopic MAP B-E, while mice of the last group were immunized with a mixture of 40 μg per mouse of MAP B-E and 20 μg per mouse of MAP C.

For the first and second immunizations, MAPs were emulsified in complete and incomplete Freund's adjuvant (Pierce, Rockford, IL, USA), respectively, and given intramuscularly 3 weeks apart. After another 3 weeks, the mice received 50 μg of MAP intraperitoneally without adjuvant. One week after the last immunization, mice were tail bled and sera were evaluated by ELISA for antibody titer and isotype against homologous MAPs, peptides and rSG3PDH. Two to four mice from the control and immunized groups were left to rest for 4 weeks after the last immunization, and then examined for spleen lymphocyte proliferation and release of interleukin (IL)-2, IL-4 and gamma interferon (IFN-γ) following stimulation with homologous MAPs, peptides, rSG3PDH or Con A.

The remaining 6 to 8 mice from each group were challenged with 125 cercariae of *S. mansoni* by the tail exposure method, 3 weeks after the last immunization. Worms were recovered by hepatic portal perfusion of mice 7 weeks after infection. Eggs in the liver and small intestine were counted after digestion with 4% KOH, and the total egg load and the egg numbers per worm pair were calculated. Data were calculated for individual mice and as a mean $\pm$ SD for each group, and the statistical significance of value differences between the test and control groups were evaluated by the Student's t-test and inferred as $p < 0.05$.

Measurement of cellular responses. Spleen cells from 2 to 4 mice from each group were pooled and suspended at concentrations of 2 and 10×10^6/ml for the proliferative and cytokine release assays, respectively. Culture medium consisted of RPMI-1640 medium supplemented with 100 U/ml of penicillin, 100 μg/ml streptomycin, 25 mM Hepes, 2 mM L-glutamine, 1 mM sodium pyruvate (all from Gibco/BRL, Paisley, UK), 5×10^{-5} M 2-mercaptoethanol and 10% fetal calf serum (ICN, Costa Mesa, CA, USA). Spleen cells were cultured for 144 (lymphoproliferation), 48 (IL-2 and IL-4) and 72 (IFN-γ) h with peptides or MAPs (10 μg/well), rSG3PDH (1 μg/well) or Con A (0.2 μg/well). Lymphoproliferation was assessed by the CellTiter 96® AQueous One Solution for Cell Proliferation Assay (Promega, Madison, WI, USA) as recommended by the manufacturer and as detailed previously [17]. Levels of IL-2, IL-4 and IFN-γ were evaluated by a sandwich ELISA using cytokine-specific capture and biotin-labelled detecting antibodies (Pharmingen, San Diego, CA, USA). Horseradish peroxidase (HRP)-labelled streptavidin (Boehringer-Mannheim, Germany) diluted 1 : 5000 was used. The reaction was evaluated spectrophotometrically at 405 nm after adding 1-Step™ABTS (2,2′-Azidobis [3-ethylbenzothiazoline-6-sulfonic acid]-diammonium salt, Pierce, Rockford, IL, USA) substrate. The mean A_{492} of duplicate wells was converted to pg/ml of the test cytokine using standard curves constructed with the recombinant mouse cytokine as recommended by the manufacturer (Pharmingen, San Diego, CA, USA). Spleen cells from the control and immunized mice did not produce detectable levels of cytokine following culture in antigen-free medium. Therefore, spleen cells of mice, which released $\geq$5 pg/ml cytokine in response to peptide antigens, rSG3PDH or Con A were considered responders.

Measurement of humoral responses by ELISA. Wells of polystyrene plates (Costar, Cambridge, MA, USA) were coated with 0.2 μg of rSG3PDH or 5 μg of MAPs in 100 μl of carbonate/bicarbonate buffer, pH 9.6. For the coating of peptides (5 μg/well), 1.5% glutaraldehyde (Sigma Chemical Co., St Louis, MO, USA) in Dulbecco's phosphate-buffered saline was used. The wells were blocked with 1% BSA in 0.05 M phosphate-buffered saline, pH 7.1 (PBS), washed with PBS/0.05% Tween 20 (washing buffer), and incubated with 100 μl of serum diluted in washing buffer. HRP-labelled goat anti-mouse IgG (H + L) conjugate (Promega, Madison, WI, USA), which was diluted 1 : 7500 was used. HRP-labelled isotype-specific rat monoclonal antibody (mAb) to

mouse IgG_1 and IgG_{2a}, alkaline phosphatase (AKP)-labelled rat mAb to mouse IgG_{2b}, IgG_3, and IgM were purchased from Pharmingen (San Diego, CA, USA), and used diluted 1 : 1000 in washing buffer. The reactivity was estimated spectrophotometrically at 405 nm after adding 1-Step™ ABTS or p-nitrophenyl phosphate substrate (Pierce). Sera giving absorbance (*A*) values higher than the cut off value (= mean *A* of wells with serum from untreated, control mice + 3 SD) were considered positive.

RESULTS AND DISCUSSION

Synthesis of Peptides and their MAP Analogues

Peptide acids A, B, C and E were prepared according to the standard solid-phase protocols on PAM resins using Boc/Bzl strategy and DCC/HOBt activation. The high efficiency of chain assembly and steric accessibility of the growing peptide anchored on a carrier play a key role in successful peptide synthesis on a solid phase. The problem is particularly important when clustered peptides, e.g. cascade branched MAPs are being synthesized. Tam and Lu [24] described coupling difficulties associated with interchain clustering and phase transition during MAP synthesis. To avoid this problem, a pre-loaded resin was prepared with the substitution initially reduced to approx. one-third of the starting value. Briefly, the MBHA resin (3.5 g, 2 mmol, 0.57 mmol NH_2/g) was coupled for 30 min with activated Boc-Gly-OH (2 mmol, DCC/HOBt activation). The resin was thoroughly washed with DMF and DCM and dried under vacuum. Quantitative AAA yielded substitution 0.175 mmol Gly/g of the peptide-resin. The remaining unreacted free amino groups were capped by Ac_2O/pyridine/DMF (1 : 2 : 10) and two levels of Lys were built up. The [Boc-Lys(Boc)]-Lys[Boc-Lys(Boc)]-Gly-NH-resin (Lys 2.89 (3), Gly 1.0 (1), the calculated substitution approx. 0.7 mmol NH_2/g of the peptide-resin) was used for the synthesis of MAPs A, B, C and E by DCC/HOBt or BOP/DIEA activation. MAP B-E was prepared analogously, but in the second level of the core, Boc-Lys(Fmoc)-OH instead of Boc-Lys(Boc)-OH was used. The activation by HBTU/DIEA and *in situ* neutralization was used throughout the synthesis of the whole molecule, because under these conditions the resin is better solvated and the coupling yields are higher than with the classical neutralization protocol [21]. After the construction of the α-chains, the terminal amino groups were acetylated. Fmoc groups were split off from N^{ε}-amino groups of lysines by the treatment with 30% piperidine in DMF (5 + 20 min) and the ε-chains were built up by Boc/Bzl strategy. EDT (2%) was added to the TFA mixture for all Boc deprotections, because of the presence of free N^{im} Trp in the α-chains. Peptides and MAPs were cleaved and deprotected by the treatment with HF/anisole (9 : 1) mixture. Purified peptides and MAPs were characterized by amino acid composition, RP-HPLC and FAB MS or MALDI TOF MS.

Purification of MAPs

Tetravalent MAPs of molecular weight above 5000 Da consist of at least 50 amino acids. Because of their branched character and high molecular weight, they cannot be treated as ordinary linear peptides but rather they should be considered as small proteins. In addition, any deletion/modification has a tremendous impact on the complexity of the crude mixture and further complicates the purification (Table 2). Assuming that any alternation in a primary sequence has, for a linear peptide, a hypothetical factor influencing separation equal to one. For a branched tetravalent MAP, this factor is reduced to one-quarter and further influenced by the position of the alternation in the peptide chain. The authors think that the use of columns with long alkyl chains and pore size around 100 Å should be avoided and suggest using C_4 columns with the pore size around 300 Å to eliminate the size exclusion effect. To prove the feasibility of RP-HPLC approach, a purification of MAP E and MAP B is given here in detail.

Purification of MAP E

The analytical HPLC profiles of the crude MAP E under various conditions on C_4 column are displayed in Figure 1. The main peak was identified

Table 2 The Impact of the Number of Deletions/Modifications on the Complexity of the Branched Peptides (Theoretical Maximal Numbers)

No. of deletions—modifications	Linear	4 valent MAP	8 valent MAP
1	1	4	8
2	2	16	64
3	3	64	512
4	4	256	4096

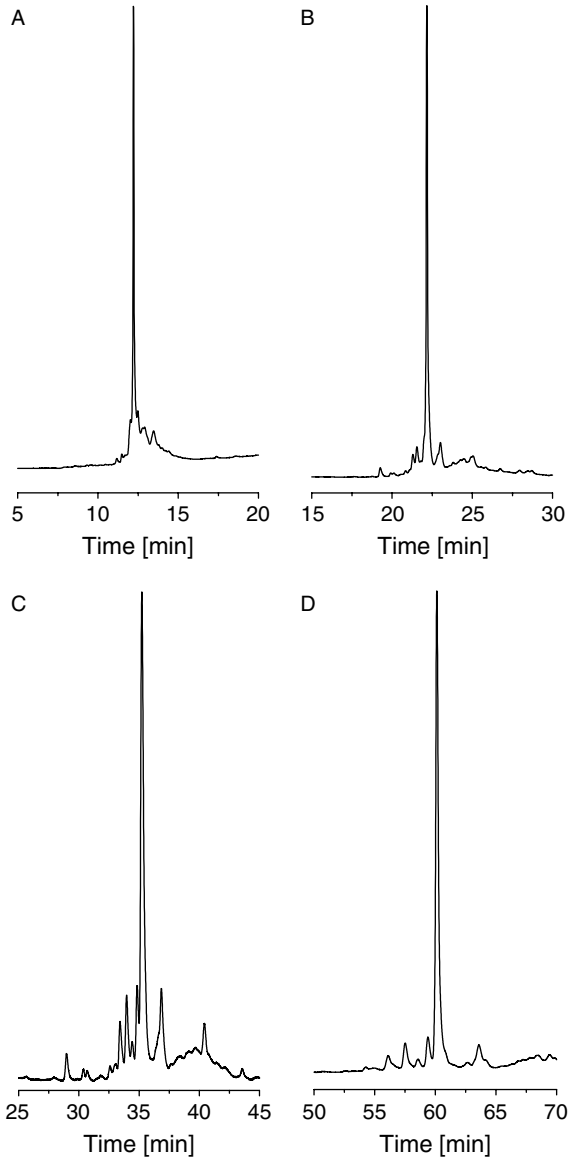

Figure 1 Analytical HPLC profiles of crude MAP E on Vydac C4 column (water–acetonitrile, 0.1% TFA v/v). **A**: 3.3% gradient, **B**: 1% gradient, **C**: 0.5% gradient, **D**: 0.25% gradient.

by MALDI-TOF MS as the product. Slowing down the gradient had a beneficial effect on the separation of the individual peaks. The replacement of acetonitrile for methanol or tetrahydrofurane had a deteriorating effect of the resolution (data not shown). The MAP E was purified on the semipreparative Vydac C_4 column using shallow 0.2% acetonitrile gradient (both phases contained 0.1% TFA v/v), flow rate 3.7 ml/min and detection at 225 nm. After lyophilization, the product was thoroughly analysed by the RP-HPLC, HPCE and MALDI-TOF MS (Figure 2). MAP E was found to be analytically pure by both HPLC and HPCE methods, with assessed purity above 95%. Mass found: 5140.71 (calculated: 5140.1), HPCE: $R_t = 2.103$ min (BGE 50 mM Tris + 100 mM H_3PO_4, pH = 2.25), HPLC: $R_t =$ 60.13 min (0.25% MeCN gradient, starting from 1% of solvent B, both phases contained 0.1% TFA).

Purification of MAP B

The purification of MAP B was not as straightforward process as for MAP E, mainly due to the significantly higher complexity of the crude product. The analytical HPLC profiles of the crude MAP B under various conditions on the C_4 column are displayed in Figure 3. Three main peaks were examined and the first one was found to contain our product. The other two contained mass −18 and −36 m.u., respectively, corresponding to the loss of one and two water molecules, probably due to aspartimide formation or cyclization of the *N*-terminal glutamic acid residue to the pyroglutamic acid residue, known side-reactions during the HF cleavage [22]. The crude MAP B was purified on a Vydac C_4 column using 0.2% acetonitrile gradient (both phases contained 0.1% TFA v/v). Only the top of the first peak was collected and analysed by HPCE. HPCE showed approx. 85% purity and revealed three minor impurities. MALDI-TOF check revealed masses differing in approx. 400 a.m.u. that were attributed to the ERD deletion peptides generated by the fission of Asp-Pro bond, a well known phenomena. Therefore, it was decided to repurify our product. A 0.08% gradient was used and TFA was replaced for AcOH in order to change the pH of the eluting buffer and therefore the selectivity. The collected peak was lyophilized and characterized by MALDI-TOF MS, HPCE and HPLC (Figure 4). MALDI-TOF identified EDR-deletion peptide still to be present as a minor impurity. The assessed purity was found to be >92% which was acceptable for the study. Mass found: 6207.14 (calculated: 6208.8), HPCE: $R_t = 2.435$ min (BGE 50 mM Tris + 100 mM H_3PO_4, pH = 2.25), HPLC: $R_t = 92.49$ min (0.25% MeCN gradient, starting from 1% of solvent B, both phases contained 0.1% TFA).

Immunogenicity and Protective Capacity of MAPs A, B, C and E

Spleen cells from the control and MAP-immunized mice showed a highly significant lymphoproliferation and production of IL-2 (approximately 60

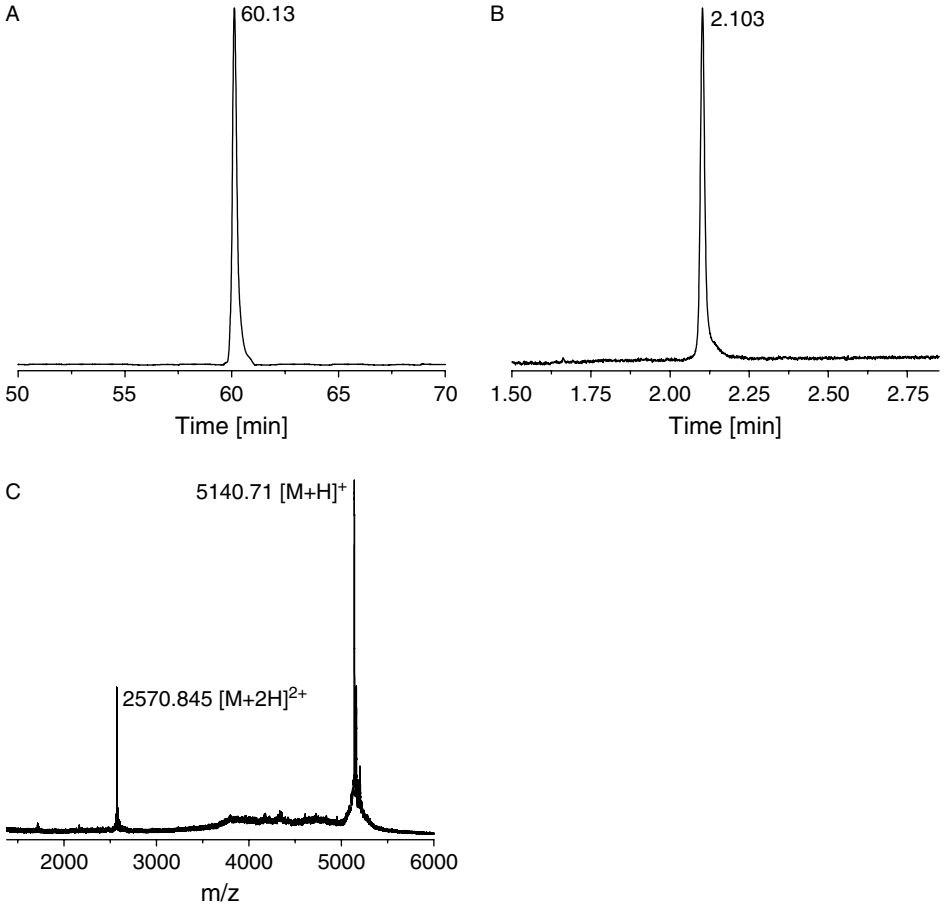

Figure 2 RP-HPLC (A) and HPCE (B) profiles and MALDI-TOF MS (C) spectrum of purified MAP E. **A**: Vydac C_4 (4.6 × 250 mm) 0.25% water–acetonitrile gradient; **B**: used buffer: BGE 50 mM Tris + 100 mM H_3PO_4, pH = 2.25; **C**: CHCA matrix, reflectron mode, MW calculated (5140.1 a.m.u.).

pg/ml), IL-4 (approx. 15 pg/ml) and IFN-γ (525 to 850 pg/ml) in response to Con A. These data indicate that MAP immunization does not impair the host immune responsiveness. Spleen cells from C57BL/6 mice immunized with MAPs A, B and C responded by significant lymphoproliferation and production of IFN-γ following restimulation *in vitro* with the homologous MAP or peptide or rSG3PDH. The findings are in accord with our results in BALB/c mice, and indicate that MAP constructs are capable of inducing significant cell-mediated immune responses to the unit peptide and cognate protein [17]. Spleen cells from mice immunized with MAP E did not proliferate significantly or produced IFN-γ in response to MAP E or peptide E (Table 3). In contrast to BALB/c mice, MAP E is not immunogenic in C57BL/6 mice, corroborating our previous observation that rSG3PDH-immunized C57BL/6 mice do not recognize peptide E [14].

Additionally, no detectable levels of IL-2 or IL-4 were found, except for 7 pg/ml of IL-4 in supernatants of cultures from MAP C-immunized mice stimulated *in vitro* with MAP C or peptide C or rSG3PDH. Similar results were recorded in BALB/c mice and suggest that SG3PDH-derived peptides fail to elicit the release of IL-4 [17].

Except for MAP E, immunization of C57BL/6 mice with MAPs elicited detectable humoral immune responses against the homologous MAP and peptide and rSG3PDH, the antibodies produced being essentially of the IgG2a, IgG2b and IgM isotype (Table 4). The antibody isotype and cellular immune responses were thus predominantly of the Th1 type. It is of note that of the different MAPs tested, MAP B elicited the most significant antibody response (Figure 5).

As for BALB/c mice, immunization of C57BL/6 mice with MAP A was not associated with significant

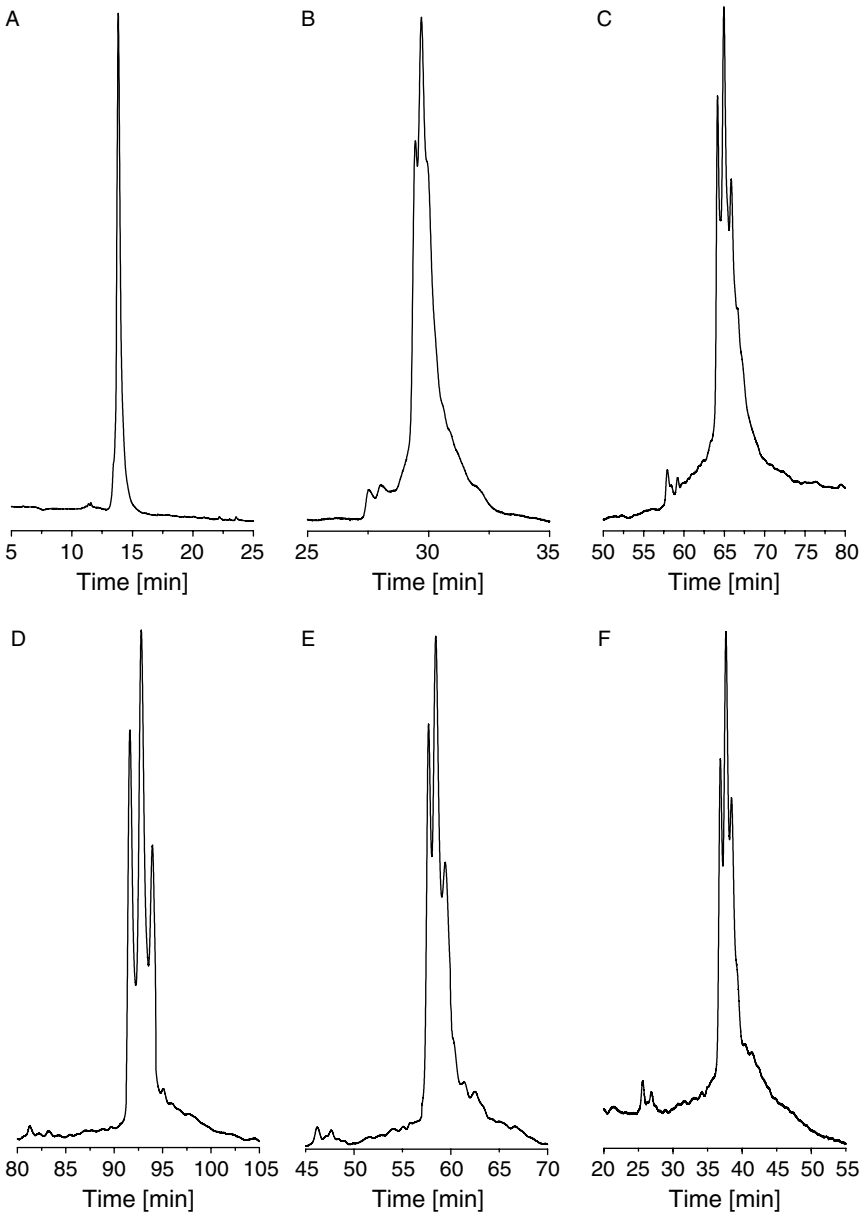

Figure 3 Analytical HPLC profiles of crude MAP B on Vydac C4 column (water–acetonitrile, 0.1% TFA v/v). **A**: 3.3% gradient, **B**: 1% gradient, **C**: 0.5% gradient, **D–F**: 0.25% gradient (starting concentration of solvent B: 1%, 10%, 15%).

changes in challenge worm burden or fecundity. Both MAP C, which elicited moderate cellular and humoral specific immune responses and MAP E, which did not elicit detectable immune responses, failed to affect the challenge worm burden and fecundity. MAP B elicited significant cellular and humoral immune responses, which were associated with a barely significant ($p = 0.051$) protection of 35% against challenge infection. The MAP B-induced protection in C57BL/6 mice and BALB/c [17] mice was higher than that induced by whole SG3PDH [14]. Our studies in BALB/c and C57BL/6 mice thus suggest peptide B can be included in a multipeptidic vaccine for protection of genetically diverse mice against schistosomiasis.

Immunogenicity and Protective Capacity of Bis-diepitopic MAP B-E and MAP B-E + MAP C

Spleen cells from mice immunized with bis-diepitopic MAP B-E or MAP B-E + MAP C responded by significant lymphoproliferation and release of

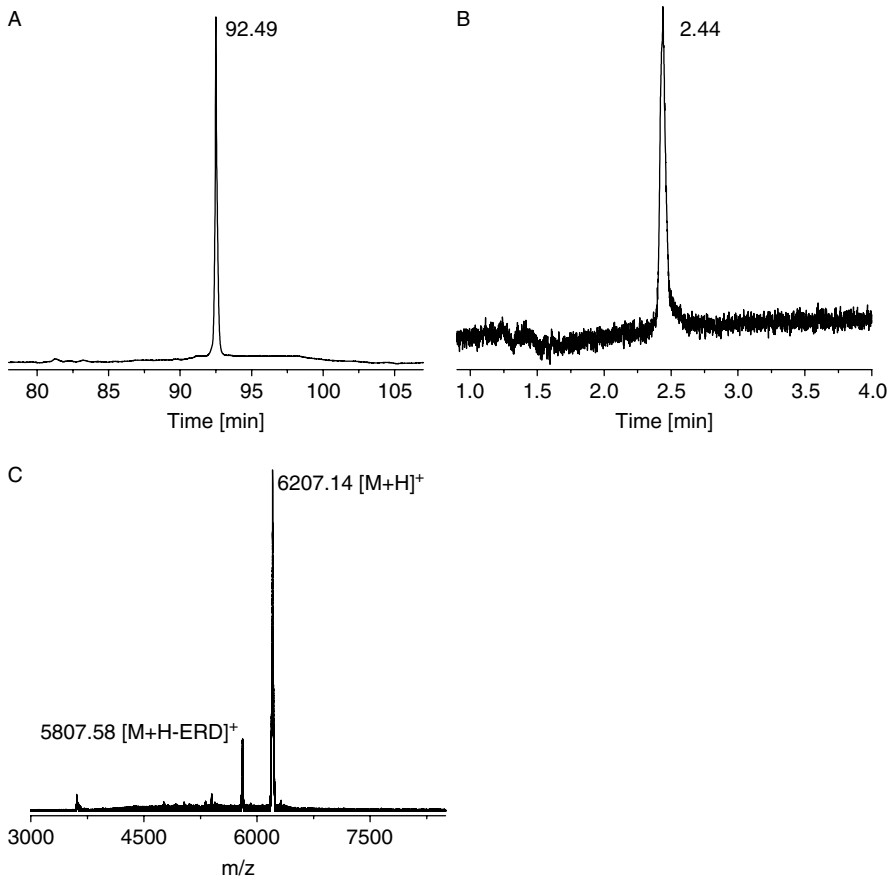

Figure 4 RP-HPLC (A) and HPCE (B) profiles and MALDI-TOF MS (C) spectrum of purified MAP B. **A**: Vydac C_4 (4.6 × 250 mm) 0.25% water–acetonitrile gradient, starting from 1% of MeCN; **B**: used buffer: BGE 50 mM Tris + 100 mM H_3PO_4, pH = 2.25; **C**: CHCA matrix, linear mode, mw calculated (6208.8 a.m.u.).

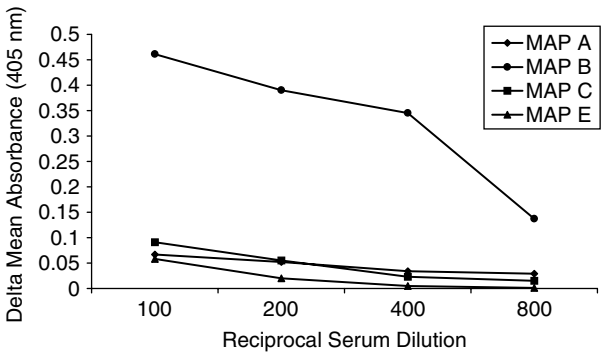

Figure 5 Titer reactivity of C57BL/6 anti-MAP sera against homologous MAPs. Sera from control and MAP-immunized C57BL/6 mice were tested against the homologous MAP by ELISA. HRP-labelled goat anti-mouse IgG (H + L) conjugate (Promega) was used diluted 1 : 7500. Each point represents delta mean absorbance = mean absorbance of sera from 8 individual mice-mean absorbance of sera from 6 control mice; SD was less than 5% of the mean.

IFN-γ to *in vitro* stimulation with MAP B or peptide B but not to MAP E or peptide E. Spleen cells from mice immunized with bis-diepitopic MAP B-E + MAP C displayed significant lymphoproliferation and IFN-γ release in response to both MAP C and peptide C (data not shown).

Sera from all mice in the two groups immunized with bis-diepitopic MAP B-E produced antibodies of titer 1 : 100–1 : 800 that bound to bis-diepitopic MAP B-E, peptide B and rSG3PDH. Only 3 of 20 sera tested (10 for each group) had antibodies specific to peptide E with titer 1 : 100. Sera of 4 of 10 mice immunized additionally with MAP C had antibodies that bound to MAP C and peptide C up to a titer of 1 : 400.

The results thus indicate that MAP B was the essential immunogen for C57BL/6 mice. Expectedly, the level of protection against challenge infection was similar in the mice immunized with bis-diepitopic MAP B-E and with or without MAP C.

Table 3 Cellular Responsiveness of C57BL/6 Mice Immunized with MAPs

Mean $A_{492} \pm$ SD of stimulated cultures and significance of differences between mean A of control and stimulated spleen cell cultures[a]						
Spleen cells from mice		Medium	MAP	Peptide	rSG3PDH	Con A
Adjuvant						
	A_{492}	0.046 ± 0.003	0.061 ± 0.009	0.040 ± 0.021	0.039 ± 0.005	0.288 ± 0.000
	$p =$		NS	NS	NS	0.0001
	IFN-γ	(ND[b])	(ND)	(ND)	(ND)	(850)
MAP A						
	A_{492}	0.149 ± 0.004	0.262 ± 0.011	0.276 ± 0.012	0.327 ± 0.050	0.608 ± 0.036
	$p =$		0.0069	0.0058	0.0392	0.0033
	IFN-γ	(ND)	(5)	(5)	(15)	(600)
MAP B						
	A_{492}	0.053 ± 0.019	0.133 ± 0.021	0.117 ± 0.019	0.159 ± 0.026	0.633 ± 0.014
	$p =$		0.0500	0.0500	0.0451	0.0008
	IFN-γ	(ND)	(7)	(7)	(15)	(525)
MAP C						
	A_{492}	0.376 ± 0.007	0.420 ± 0.005	0.496 ± 0.019	0.599 ± 0.052	0.877 ± 0.031
	$P =$		0.0213	0.0144	0.0271	0.0021
	IFN-γ	(ND)	(30)	(30)	(15)	(650)
MAP E						
	A_{492}	0.078 ± 0.022	0.190 ± 0.102	0.161 ± 0.034	0.596 ± 0.331	0.594 ± 0.039
	$p =$		NS	NS	NS	0.0039
	IFN-γ	(ND)	(ND)	(ND)	(ND)	(620)

[a] Mean absorbance (A_{492}) of triplicate control and test cultures and SD about the mean were statistically analysed using the Student's *t*-test. NS = not significant.
[b] Levels of IFN-γ in pg/ml are given in parentheses. ND, not detectable.

Table 4 Serum IgG Isotype Response of C57BL/6 Mice Immunized with MAPs

Antibody isotype response (fold increase of immune over control serum)[a] against			
Sera from mice immunized with	Homologous MAP	Homologous peptide	rSG3PDH
MAP A	IgG_{2a} (1.9)	IgG_{2a}, IgG_{2b} (2.0, 1.85)	IgG_{2a}, IgG_{2b} (10.0, 7.1)
MAP B	IgG_{2a}, IgG_{2b} (8.4, 7.6)	IgG_{2a}, IgG_{2b} (4.2, 2.0)	IgG_{2a}, IgG_{2b} (5.2, 5.8)
MAP C	IgG_{2b} (2.2)	IgG_{2b} (2.0)	IgG_{2b} (2.7)
MAP E	ND[b]	ND	ND

[a] Fold increase of immune over control serum is given between parentheses.
[b] MAP E induced only specific IgM antibodies; IgG antibodies were not detected (ND).

In both groups of mice, a significant ($p < 0.05$) decrease in worm burden was corroborated by a significant ($p < 0.05$) decrease in egg load. However, no effect on worm fecundity (egg count/worm couple) was recorded (Table 5).

The studies thus confirm that SG3PDH-derived peptide B is strongly immunoprotective. Therefore, it is useful to use this peptide within a polyvalent construct containing similarly well-characterized peptides from other schistosome candidate molecules.

CONCLUSION

MAP constructs elicit cellular and humoral immune responses to the immunogen, the unit peptide and cognate protein. In contrast to BALB/c mice, MAP E was poorly immunogenic in C57BL/6 mice, supporting the well-established genetic restrictions in peptide-mediated immune responses. MAP B elicited significant cellular and humoral immune

Table 5 Worm and Egg Burden in C57BL/6 Mice Immunized with MAPs

Mice immunized with	Worm and egg burden (mean ± SD)	$p =$[a]	Percent reduction[b]
Worm burden			
Negative controls	76.6 ± 28.0		
MAP B-E	36.4 ± 28.0	0.034	52.4
MAP B-E + MAP C	40.6 ± 13.6	0.021	46.9
Egg count/mouse			
Negative controls	12490 ± 7383		
MAP B-E	5251 ± 1744	0.043	57.9
MAP B-E + MAP C	5244 ± 2513	0.049	58.0
Egg count/worm couple			
Negative controls	329 ± 106		
MAP B-E	289 ± 88	NS	
MAP B-E + MAP C	258 ± 37	NS	

[a] Statistical significance of value differences between test and control groups examined six weeks after infection was evaluated by the Student's *t*-test and inferred as $p < 0.05$.
[b] Percent reduction calculated according to the formula: mean number in controls — mean number in test/mean number in controls ×100.

responses in both BALB/c and C57BL/6 mice, suggesting that the peptide B may be included in a multi-peptidic schistosomiasis vaccine for genetically diverse hosts.

The purification of MAPs by the RP-HPLC methodology is given here in detail. It is shown that it is possible to obtain analytically pure compounds even for branched molecules with a molecular weight exceeding 6000 Da. Of course, the quality of the synthesis, intrinsic properties of the peptide sequence, the nature of deletions/modifications, the choice of column and eluting conditions are determining factors affecting purification.

Acknowledgements

The authors would like to express their thanks to Mr J. Zbrožek for amino acid analyses, J. Cvačka, PhD for MALDI-TOF MS measurements and V. Kašička, PhD for capillary electrophoresis. This work was supported by the Research Project CEZ:AV0Z4055905, Grant Agency of the Czech Republic (grant no. 303/01/0690 to J. J.) and by ICGEB (grant no. CRP/EGY98-03 to R. R.).

REFERENCES

1. Velek J, Ježek J, Velková V, Montash M, Tallima H, El Ridi R. Immunogenicity and immunoprotective capacity of synthetic peptides from *Schistosoma mansoni* glyceraldehyde 3-phosphate dehydrogenase. *Collection Symp. Series* 2001; **4**: 13–16.
2. Ježek J, Velek J, Velková V, Montash M, Tallima H, El Ridi R. Synthetic peptides and multiple antigenic peptides derived from *Schistosoma mansoni* glyceraldehyde 3-phosphate dehydrogenase: Immunogenicity, antigenicity and protective capacity. In *7th International Symposium 'Solid Phase Synthesis and Combinatorial Chemical Libraries', Southampton, UK, 2001*, Epton R (ed.). Mayflower Worldwide: Kingswinford, UK, 2003; 213–216.
3. Chitsulo L, Engels D, Montresor A, Savioli L. The global status of schistosomiasis and its control. *Acta Trop.* 2000; **77**: 41–51.
4. Dunne D, Mountford A. Resistance to infection in humans and animal models. In *Schistosomiasis*, Mahmoud AAF (ed.). Imperial College Press: London, 2001; 133–212.
5. Dessein AJ, Begley M, Demeure C, Caillol D, Fueri J, Reis MG, Andrade ZA, Prata A, Bina JC. Human resistance to *Schistosoma mansoni* is associated with IgG reactivity to a 37-kD larval surface antigen. *J. Immunol.* 1988; **140**: 2727–2736.
6. Goudot-Crozel V, Caillol D, Djabali M, Dessein AJ. The major parasite surface antigen associated with human resistance to schistosomiasis is a 37-kD glyceraldehyde-3P-dehydrogenase. *J. Exp. Med.* 1989; **170**: 2065–2080.
7. El Ridi R, Farouk F, Sherif M, Al-Sherbiny M, Osman A, El Gengehi N, Shoemaker CB. T and B cell reactivity to a 42-kDa protein is associated with human resistance to both *S. mansoni* and *haematobium*. *J. Infect. Dis.* 1998; **177**: 1364–1372.
8. El Ridi R, Shoemaker CB, Farouk F, El Sherif NH, Afifi A. Human T- and B-cell responses to *Schistosoma mansoni* glyceraldehyde 3-phosphate dehydrogenase correlate with resistance to reinfection with *S. mansoni* or *S. haematobium* after chemotherapy. *Infect. Immun.* 2001; **69**: 237–244.
9. Bergquist R, Al-Sherbiny M, Barakat R, Olds R. Blueprint for schistosomiasis vaccine development. *Acta Trop.* 2002; **82**: 183–192.
10. Argiro L, Kohlstadt S, Henri S, Dessein H, Matabiau V, Paris P, Bourgois A, Dessein AJ. Identification of a candidate vaccine peptide on the 37kDa *Schistosoma mansoni* GAPDH. *Vaccine* 2000; **18**: 2039–2048.
11. Wolowczuk I, Auriault C, Gras-Masse H, Vendeville C, Balloul J-M, Tartar A, Capron A. Protective immunity in mice vaccinated with the *Schistosoma mansoni* P-28-1 antigen. *J. Immunol.* 1989; **142**: 1342–1350.
12. Reynolds SR, Shoemaker CB, Harn DA. T and B cell epitope mapping of Sm23, an integral membrane

protein of *Schistosoma mansoni*. *J. Immunol.* 1992; **149**: 3995–4001.
13. Reynolds SR, Dahl CE, Harn DA. T and B epitope determination and analysis of multiple antigenic peptides for the *Schistosoma mansoni* experimental vaccine triose-phosphate isomerase. *J. Immunol.* 1994; **152**: 193–200.
14. El Ridi R, Mahrous A, Afifi A, Montash M, Velek J, Ježek J. Human and murine humoral immune recognition of multiple peptides from *Schistosoma mansoni* glyceraldehyde 3-P dehydrogenase is associated with resistance to schistosomiasis. *Scand. J. Immunol.* 2001; **54**: 477–485.
15. Tam JP. Synthesis and applications of branched peptides in immunological methods and vaccines. In *Peptides: Synthesis, Structures, and Applications*, Gutte B (ed.). Academic Press Inc.: San Diego, 1995; 455–500.
16. Zegers ND, Claassen E, Boersma WJA. Multiple antigen peptides for immunization. In *Immunological Recognition of Peptides in Medicine and Biology*, Zegers ND, Boersma WJA, Claassen E (eds). CRC: Boca Raton, FL, 1995; 205–212.
17. Tallima H, Montash M, Vepřek P, Velek J, Ježek J, El Ridi R. Differences in immunogenicity and vaccine potential of peptides from *Schistosoma mansoni* glyceraldehyde 3-phosphate dehydrogenase. *Vaccine* 2003; **21**: 3290–3300.
18. Spetzler JC, Tam JP. Unprotected peptides as building blocks for branched peptides and peptide dendrimers. *Int. J. Peptide Protein Res.* 1995; **45**: 78–85.
19. Basak A, Boudreault A, Chen A, Chrétien M, Seidah NG, Lazure C. Application of the multiple antigenic peptides (MAP). Strategy to the production of prohormone convertases antibodies: synthesis, characterization and use of 8-branched immunogenic peptides. *J. Peptide Sci.* 1995; **1**: 385–395.
20. Kaiser E, Colescott RL, Bossinger CD, Cook PI. Color test for the detection of free terminal amino groups in the solid-phase synthesis of peptides. *Anal. Biochem.* 1970; **34**: 595–598.
21. Ježek J, Houghten RA. A comparative study of BOP as a coupling agent using simultaneous multiple peptide synthesis. *Collect. Czech. Chem. Commun.* 1994; **59**: 691–706.
22. Tam JP, Merrifield RB. Strong acid deprotection of synthetic peptides: Mechanisms and methods. In *The Peptides. Analysis, Synthesis, Biology*, Vol. 9, Part C, Udenfriend S, Meienhofer J, (eds). Academic Press: New York, 1987; 185–248.
23. Kašička V, Prusík Z, Sázelová P, Brynda E, Stejskal J. Capillary zone electrophoresis with electroosmotic flow controlled by external radial electric field. *Electrophoresis* 1999; **20**: 2484–2492.
24. Tam JP, Lu YA. Coupling difficulty associated with interchain clustering and phase transition in solid phase peptide synthesis. *J. Am. Chem. Soc.* 1995; **117**: 12 058–12 063.

