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Monoclonal antibodies against AsaP1, a major exotoxin of the fish pathogen *Aeromonas salmonicida* subsp. *achromogenes*, and their application in ELISA

U. Wagner[1], B.K. Gudmundsdóttir[2] and K. Drössler[1]

[1]Institute for Zoology, University of Leipzig, Germany, and [2]Institute for Experimental Pathology, University of Iceland, Keldur, Reykjavík, Iceland

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U. WAGNER, B.K. GUDMUNDSDÓTTIR AND K. DRÖSSLER. 1999. Two monoclonal antibodies (Mabs) binding to a toxic extracellular metallo-proteinase of *Aeromonas salmonicida* subsp. *achromogenes*, AsaP1, were produced. Both reacted with common epitopes of the native enzyme and recognized this 20 kDa antigen on Western blots. One of these Mabs had an inhibitory effect on the caseinase activity of the exotoxin. A Mab-based ELISA was set up and evaluated for serological detection of AsaP1 in bacterial culture filtrates. The exotoxin was identified serologically in the extracellular products of 11 of 26 atypical *Aer. salmonicida* isolates, including the type strain for subsp. *achromogenes* NCIMB 1110. The ELISA was approximately 100-fold more sensitive in detecting AsaP1 compared with an azocasein assay. The established serological test enables AsaP1 to be quantified reliably with a lower detection limit of about 0·12 ng ml^{-1} and has a potential use for the phenotypic differentiation of atypical *Aer. salmonicida* isolates.

INTRODUCTION

'Typical' *Aeromonas salmonicida* (subsp. *salmonicida*) strains are known to be the causative agent of classical furunculosis of salmonids and some other fish species, but 'atypical' *Aer. salmonicida* (subsp. *achromogenes*, subsp. *masoucida*, subsp. *smithia*, and strains that do not fit into the existing classification) are associated with diverse ulcerative diseases in a variety of fish species. The extracellular products (ECPs) of typical and atypical *Aer. salmonicida* strains are known to differ markedly (Ellis 1991; Austin and Austin 1993; Gudmundsdóttir 1996; Wiklund and Dalsgaard 1998). Correlation has been demonstrated between pathogenic properties of atypical *Aer. salmonicida* ECPs and proteolytic exoenzymes (Gunnlaugsdóttir and Gudmundsdóttir 1997). Different profiles of exoproteinases may be a useful tool for the intraspecific classification of atypical strains, which are known to be biochemically and genotypically more diverse than the typical strains (Gudmundsdóttir 1996; Austin *et al*. 1998).

Correspondence to: Dr Ulrich Wagner, Institute for Zoology, University of Leipzig, Talstr. 33, D–04103 Leipzig, Germany (e-mail: olli@rz.uni-leipzig.de).

The major extracellular toxin of the type strain for *Aer. salmonicida* subsp. *achromogenes*, NCIMB 1110, and a group of 'atypical' strains, is a caseinolytic 20 kDa metallo-proteinase (AsaP1) possessing an LD_{50} of 0·03 μg protein g^{-1} for juvenile Atlantic salmon (*Salmo salar* L.) (Gudmundsdóttir *et al*. 1990). Recent results described by Hirst and Ellis (1996) suggest that this exoenzyme may be implicated in the siderophore-independent iron acquisition of this bacterial pathogen. *In vitro*, AsaP1 was not cytotoxic but possessed immunomodulatory features (Gudmundsdóttir *et al*. 1995; Gudmundsdóttir and Gudmundsdóttir 1996). In Atlantic salmon, the specific humoral immune response against this exotoxin demonstrated a strong association with protection against a virulent bacterial challenge (Gudmundsdóttir and Magnadóttir 1997). Furthermore, its relevance as a component of efficient vaccines against *Aer. salmonicida* subsp. *achromogenes* has recently been documented (Gudmundsdóttir *et al*. 1997).

Isolates producing the AsaP1 exotoxin have so far been identified qualitatively by different analyses of extracellular proteolytic activities such as substrate specificity, inhibitor sensitivity and mobility in substrate gel electrophoresis. For the quantification of its proteolytic activity, casein or azo-

casein have been used as substrates (Gudmundsdóttir *et al.* 1990; Gudmundsdóttir 1996).

Monoclonal antibodies (Mabs) are known to be valuable tools for immunodiagnosis of fish pathogens, for structural as well as biochemical analyses of virulence relevant antigens and for the development of various ELISA techniques (Austin *et al.* 1986; Porstmann and Kiessig 1992; Morelli *et al.* 1994; Kooi and Sokol 1996; Lachmann *et al.* 1998). Furthermore, such antibodies can be helpful tools for the development of efficient vaccines (Adams *et al.* 1995).

The aim of this study was to raise and characterize Mabs to the major toxic exoproteinase of *Aer. salmonicida* subsp. *achromogenes* and to set up an ELISA suitable for serological identification of AsaP1-producing strains, as well as for quantitative antigen measurement.

MATERIALS AND METHODS

Bacterial strains

All bacterial strains used in this study are listed in Table 1. Antigens of strain 265/87 were applied to the generation of Mabs. Bacteria were stored in brain heart infusion broth (BHIB, Oxoid) or phosphate-buffered saline (PBS), pH 7·4, containing 15% (v/v) glycerol at −140 °C. Atypical strains were routinely cultured on blood agar at 22 °C. Stock cultures of typical strains were maintained on tryptone-soy-Coomassie-agar (Bernoth 1990) at 4 °C and subcultured at 4–6 week intervals.

Preparation of bacterial antigens

Extracellular products (ECPs). Atypical strains were cultivated in BHIB in a shaking water bath at 22 °C for 72 h. ECPs were obtained after centrifugation of cells at 4000 *g* for 30 min and filtration (0·22 μm membranes) of the culture supernate (B-ECP). Alternatively, ECPs were produced by the cellophane overlay method (O-ECP) as described by Gudmundsdóttir (1996). B-ECP of typical strains were obtained after cultivation in BHIB at 22 °C for 30 h. Protein concentrations were determined by the Bradford method (Bradford 1976) with bovine serum albumin as standard protein.

Biotinylation of O-ECP. O-ECP (0·25 mg) of strain 265/87 was dissolved in 2·5 ml 0·1 mol l^{-1} HEPES/HCl containing 5 mmol l^{-1} 1,10-phenanthroline (oPA) and 0·25 mmol l^{-1} PMSF, pH 8·5, treated with 1·25 mg biotinamidocaproic acid 3-sulpho-N-hydroxy-succinimide ester (Sigma) at 22 °C for 4 h and dialysed against Tris-buffered saline (TBS) containing 0·1 mmol l^{-1} oPA at 4 °C overnight. Biotinylated antigens (bioECP) were stored at −20 °C until use.

Proteinase isolation. Proteinase antigen for immunization was prepared from O-ECP of strain 265/87 by anion-exchange chromatography and gel filtration as previously described by Gudmundsdóttir *et al.* (1990). The material of peak fractions was freeze-dried, solubilized in a small amount of 40% (v/v) ethanol on ice and diluted in PBS. Possible LPS contamination was eliminated at 4 °C overnight using an END-X™ B15 Endotoxin Removal Kit (Associates of Cape Cod, Inc., Woods Hole, MA, USA).

Enzymatic analyses

Azocasein assay. Caseinolytic activity of ECP samples was measured by hydrolysis of azocasein (Sigma) based on the method of Sarath *et al.* (1989). A 100 μl aliquot of sample was incubated with 400 μl of 1% (w/v) azocasein in 0·06 mol l^{-1} phosphate buffer, pH 7·2, at 22 °C for 2 h. In reagent blanks, phosphate buffer was used instead of ECPs. Reactions were stopped by the addition of 0·5 ml 10% (w/v) trichloroacetic acid (TCA). After 30 min, precipitates were removed by centrifugation at 4000 *g* for 10 min, and a 0·5 ml aliquot of the supernate was added to 0·5 ml of 1 mol l^{-1} NaOH. Released azodye was measured spectrophotometrically at A_{450} against a reagent blank. One unit of proteolytic activity was defined as an increase in A_{450} of 0·01 under the assay conditions. The tests were performed in duplicate.

Detection of AsaP1 activity. The AsaP1 proteinase was detected in O-ECP by its PMSF-resistant and oPA-sensitive caseinolytic activity, as well as its M_r of approximately 20 kDa in substrate SDS-PAGE (Gudmundsdóttir 1996).

Hybridoma generation

A female BALB/c mouse was immunized with 40 μg AsaP1 antigen. Purified proteinase in PBS containing 10 mmol l^{-1} EDTA was emulsified with an equal volume of Titermax® (CytRx Corp., Norcross, GA, USA) as adjuvant and a total volume of 0·1 ml was given subcutaneously. Booster injections were carried out after 8 and 12 weeks, with 100 and 200 μg of antigen with adjuvant, respectively. Finally, the native antigen (20 μg in PBS) was administered intraperitoneally on two consecutive days, 12 and 24 weeks later. Splenocytes were harvested 3 days after the final antigen injection. Fusion of splenocytes, generation of hybridomas and immunoglobulin isotyping of Mabs were performed as previously described (Wagner *et al.* 1997).

ECP-capture assay

ELISA plates (Greiner, Frickenhausen, Germany) were coated with goat-antimouse IgG (Jackson ImmunoResearch

Table 1 Comparison of serological and enzymatic AsaP1 detection in the ECPs of various *Aeromonas salmonicida* strains

Strain designation	Host species and country of isolation	Contribution*	AsaP1-ELISA reactivity		AsaP1-activity§
			Mab 9F9†	Mab 8F11‡	
'Atypical' *Aer. salmonicida*					
265/87, 138/88, 17/87, S226/88	*Salmo salar*, Iceland	Fish Disease Laboratory, Keldur, Reykjavík	+	+	+
ASN	*Salmo salar*, Norway	Fish Disease Laboratory, Keldur, Reykjavík	+	+	+
909/81, 2013/81	*Salmo salar*, Norway	Fish Disease Laboratory, Fredriksberg	+	+	+
1977/88	*Salmo salar*, Norway	Fish Disease Laboratory, Fredriksberg	−	−	−
No. 1	*Salmo trutta*, Finland	Fish Disease Laboratory, Fredriksberg	−	−	−
No. 2	*Salmo trutta*, Finland	Fish Disease Laboratory, Fredriksberg	(+)	(+)	−
No. 3	*Thymallus thymallus*, Finland	Fish Disease Laboratory, Fredriksberg	−	−	−
860613-1/1	*Salmo salar*, Faroe Islands	Fish Disease Laboratory, Fredriksberg	−	−	−
920225-1/2	*Anguilla anguilla*, Denmark	Fish Disease Laboratory, Fredriksberg	−	−	−
921203-2/3	*Platichthys flesus*, Denmark	Fish Disease Laboratory, Fredriksberg	−	−	−
261/89	*Salmo trutta*, Sweden	Fish Disease Laboratory, Fredriksberg	−	−	−
298/89	*Salvelinus alpinus*, Sweden	Fish Disease Laboratory, Fredriksberg	−	−	−
150, 153, 154	*Cyprinus carpio*, Canada	Aqua Health Ltd., Charlottetown	−	−	−
86663	*Salmo salar*, Canada	Department of Microbiology, Galway	−	−	−
MT 179	*Salmo salar*, Scotland	SOAFD Marine Laboratory, Aberdeen	+	+	+
5127/88	*Salvelinus alpinus*, Finland	National Veterinary and Food Research Institute, Oulu	−	−	−
87:1147	*Cyprinus carpio*, Australia	CSIRO, Australian Animal Health Laboratory, Geelong	(+)	(+)	−
NCIMB 1110	*Salmo trutta*, Scotland	NCIMB, Type strain subsp. *achromogenes*	+	+	+
NCIMB 2020	*Oncorhynchus masou*, Japan	NCIMB, Type strain subsp. *masoucida*	−	−	−
NCIMB 13210	*Rutilus rutilus*, England	NCIMB, Type strain subsp. *smithia*	−	−	−
'Typical' *Aer. salmonicida*					
1880/95	*Salmo salar*, Norway	Department of Fish Health, Oslo	−	−	−
MT 004, MT 059	*Salmo salar*, Scotland	SOAFD Marine Laboratory, Aberdeen	−	−	−
343	*Salmo salar*, Sweden	Fish Disease Laboratory, Keldur, Reykjavík	−	−	−
F216·1/83	*Oncorhynchus mykiss*, Germany	Fish Disease Laboratory, Federal Health Office, Berlin	−	−	−
NCIMB 1102	*Salmo salar*, Scotland	NCIMB, Type strain subsp. *salmonicida*	−	−	−

* I. Dalsgaard, Fish Disease Laboratory, Danish Institute of Fisheries and Marine Research, Fredriksberg, Denmark; R.D. Osinchuk, Aqua Health Ltd, Charlottetown, Prince Edward Island, Canada; R. Powell, Department of Microbiology, University College Galway, Ireland; T.S. Hastings, SOAFD Marine Laboratory, Aberdeen, UK; V. Hirvelä-Koski, National Veterinary and Food Research Institute, Oulu, Finland; E.-M. Bernoth, CSIRO Australian Animal Health Laboratory, Geelong, Victoria, Australia; S. Høie, Department of Fish Health, Central Veterinary Laboratory, Oslo, Norway; Keldur, Institute for Experimental Pathology, Fish Disease Laboratory, University of Iceland, Reykjavík, Iceland; E.-M. Bernoth, Fish Disease Laboratory, Federal Health Office, Berlin, Germany; NCIMB: National Collection of Industrial and Marine Bacteria, Aberdeen, UK.

ELISA reactivities were expressed as difference in A_{405} values to negative controls containing the irrelevant Mab 13C7.

† −, <0·4; (+), 0·4–1·0; +, >1·0.

‡ −, <0·7; (+), 0·7–1·3; +, >1·3.

§ +, AsaP1 was demonstrated as an oPA-sensitive caseinolytic activity with a M_r of approximately 20 kDa in substrate SDS-PAGE.

Lab., West Grove, PA, USA; 5 μg ml^{-1} in PBS, 50 μl per well) for 20 h at 22 °C and incubated with hybridoma supernate (diluted 1 in 3 in PBS/0·1% [v/v] Tween-20, PBS/T) at 4 °C overnight. After three washing steps with ice-cold PBS/T, wells were exposed to bioECP (diluted 1 in 50 in PBS/T, 2 h, 4 °C) and streptavidin biotinylated peroxidase complexes (Amersham; 1 in 1000, 1·5 h, 4 °C). Reactions were monitored colorimetrically using 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid) diammonium salt (ABTS) as a chromogen and was read at A_{405} after 1 h at 22 °C.

Proteinase-capture assay

Goat-antimouse IgG coated ELISA plates were incubated successively with hybridoma supernate (50 μl per well, diluted 1 in 3 in TBS/0·1% [v/v] Tween-20, TBS/T), freshly prepared B-ECP of strain 265/87 (diluted 1 in 2 in TBS/T) and proteinase substrate (Wagner *et al*. 1997). Substrate solutions were prepared by dissolving 40 mg casein (according to Hammarsten, Merck) or 50 mg gelatin from porcine skin (Sigma) in 50 ml TBS at 50 °C, followed by the addition of 25 ml 0·2 mol l^{-1} Tris/HCl, 10 mmol l^{-1} $CaCl_2$, pH 9·0, and filter sterilization (0·22 μm). Substrate digestion (75 μl per well) was terminated after an overnight incubation at 37 °C by the addition of 20% (w/v) TCA (100 μl per well). Reactions were measured spectrophotometrically at A_{570} after 15 min incubation at 22 °C. Mab 9D5 (IgG1, K) recognizing the 70 kDa serine proteinase (P1, AspA) of typical strains (Wagner *et al*. 1997) as well as the irrelevant Mab 13C7, binding to L(–)-carnitine dehydratase of *Escherichia coli* (Preusser *et al*. 1999), were used as control Mabs for the assay.

Western blotting

Extracellular antigens were immunoblotted by a Western blotting test as previously described (Wagner *et al*. 1997). O-ECP of strain 265/87 was employed as the antigen preparation, hybridoma supernates were used to probe the blotted antigens and anti-AspA-Mab 9D5 was used to control the specificity of the reaction. Following transfer, the nitrocellulose membranes were stained for total proteins with Ponceau S (Serva; 0·2 mg ml^{-1} in 2% [v/v] acetic acid) and destained with PBS.

Immunoprecipitation experiments

Latex beads (2·9 μm, Polysciences, Warrington, PA, USA) were coated with goat-antimouse IgG and successively incubated with hybridoma supernates and B-ECP as previously described (Wagner *et al*. 1997). Precipitated antigens were separated by SDS-PAGE under reducing conditions (Laemmli 1970). Proteins were silver-stained with Bio-Rad silver stain plus kit according to the manufacturer's instructions, or analysed by Western blotting with a rabbit antiserum (Ranti-AsaP1, 1 in 1000) raised against the AsaP1 proteinase isolated from strain 265/87 (Gudmundsdóttir and Magnadóttir 1997).

Enzyme inhibition studies

Hybridoma supernates containing different Mabs were treated with 1 mmol l^{-1} PMSF and concentrated approximately four times using Microcon®–100 microconcentrators (Amicon). Immunoglobulin concentrations were determined by ELISA with a mouse IgG1 standard (Southern Biotechnology Associates, Birmingham, AL, USA). B-ECP (50 μl) of strain 265/87 were incubated with 50 μl of these Mab preparations, concentrated hybridoma culture medium, or 0·06 mol l^{-1} phosphate buffer, pH 7·2, at 4 °C for 2 h. Control B-ECP samples were treated with 1 mmol l^{-1} PMSF or 4 mmol l^{-1} oPA. Proteolytic activity of reaction mixtures was then analysed by the azocasein assay.

Mab-based AsaP1-ELISA

Goat-antimouse IgG (5 μg ml^{-1}, 50 μl per well) coated ELISA plates were incubated with Mab-containing hybridoma supernates (diluted 1 in 10 in PBS/T) at 4 °C overnight and washed three times with ice-cold PBS/T. For detection of AsaP1, wells were filled with 50 μl of either O-ECP (5 μg protein ml^{-1} in PBS/T) or B-ECP (diluted 1 in 5 in ice-cold PBS/T; $\leqslant$4 μg protein ml^{-1}) and incubated at 4 °C for 2 h. After three washing steps, Mab-captured antigen was detected with a rabbit antiserum against formalin-inactivated O-ECP of isolate 265/87 (Ranti-f ECP265/87, 1 in 4000, 2 h, 4 °C) and peroxidase-conjugated goat-antirabbit IgG (Jackson ImmunoResearch Lab.; 1 in 10000, 1·5 h, 4 °C). ABTS was used as a chromogen and A_{405} was read after 60 min at 22 °C. In order to establish the specificity of the AsaP1-ELISA, an isotype-matched irrelevant Mab (13C7), Mabs recognizing the AspA-and P2-exoproteinases of the typical strain F216·1/83 (9D5, 3B11), and a rabbit serum (R-a-F216, 1 in 4000) raised against B-ECPs of this strain (Wagner *et al*. 1997), were employed. Imprecision of the ELISA was assessed with aliquots of an O-ECP sample, stored at –70 °C until use. The detection limit of the test was defined as the lowest protein concentration giving a significant ELISA signal. As a cut-off value, the mean absorbance value + 5 × S.D. of a zero-dose sample was used. Intra- and inter-assay variability was determined as described by Porstmann and Kiessig (1992), with the exception that 10-fold determinations for each analysis were used.

RESULTS

Isolation and characterization of monoclonal antibodies

Hybridomas producing Mabs against strain 265/87-secreted antigens were selected by the ECP-capture assay. Eight of 240

cell clones gave positive reactions. When tested by Western blotting, antibodies of two hybridomas, designated 9F9 and 8F11, reacted with a 20 kDa antigen (Fig. 1). Immunoprecipitation experiments, carried out to ascertain the M_r of the antigen captured by these two antibodies from native B-ECP, emphasized the recognition of a protein of about 20 kDa (Fig. 2). In the proteinase-capture assay, molecules trapped by the Mabs possessed weak caseinolytic activity, whereas gelatinolytic properties were not detectable (Fig. 3). These characteristics indicate the AsaP1 specificity of Mabs 9F9 and 8F11. Both Mabs were of the IgG1 subclass with κ light chains.

Enzyme inhibition studies revealed that Mab 9F9 lowered the caseinolytic activity of strain 265/87 B-ECP by about 15%, but Mab 8F11 and three control Mabs (13C7, 9D5, 3B11) showed no inhibitory effect (Fig. 4). The recorded proteolytic activity was severely inactivated by 4 mmol l^{-1} oPA but not by PMSF. The immunoglobulin concentration of the Mab preparations used varied between 85 μg ml^{-1} (Mab 3B11) and 120 μg ml^{-1} (Mab 8F11). No caseinolytic

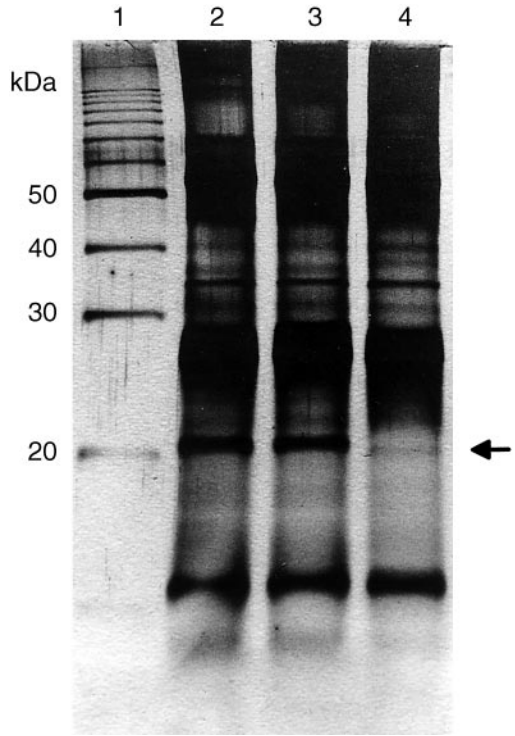

Fig. 2 SDS-PAGE analysis of antigens immunoprecipitated from B-ECP of strain 265/87 by Mab 8F11 (lane 2), 9F9 (lane 3) and Mab 13C7 as negative control (lane 4). Marker proteins (Gibco) were separated in lane 1. The band indicated by an arrow could be immunostained following Western blotting by a rabbit anti-AsaP1-serum (Ranti-AsaP1). Strongly stained material of approximately 50 and 25 kDa represent immunoglobulin H- and L-chains, respectively

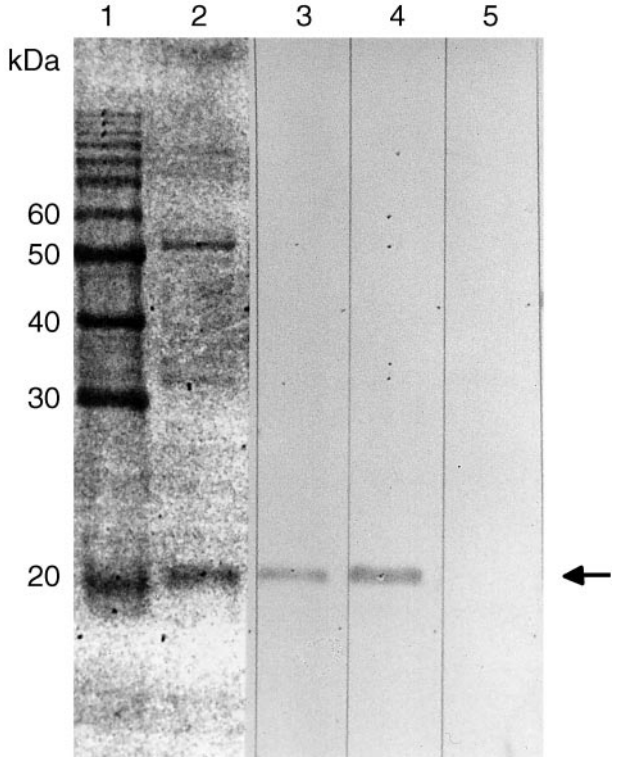

Fig. 1 Reactivity of monoclonal antibodies against immunoblotted extracellular antigens of *Aeromonas salmonicida* subsp. *achromogenes*, strain 265/87 (O-ECP; 2·5 μg per lane). Ponceau-staining of marker proteins (Gibco; lane 1) and O-ECP (lane 2); immunostaining of O-ECP with Mab 9F9 (lane 3), Mab 8F11 (lane 4) and control Mab 9D5 (lane 5). The AsaP1 caseinase is indicated by an arrow

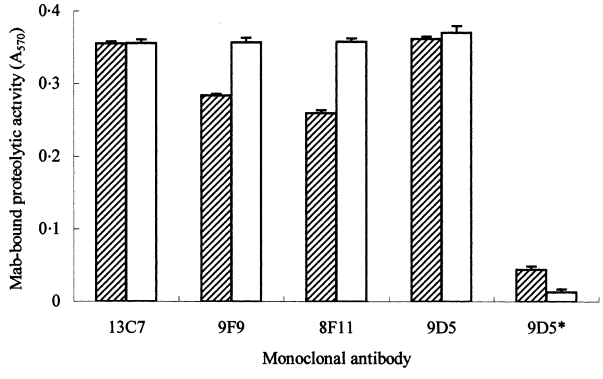

Fig. 3 Proteolytic activity of Mab-bound antigens studied by the proteinase-capture assay and B-ECP of *Aeromonas salmonicida* subsp. *achromogenes*, strain 265/87, as antigen preparation. Digestion of employed substrates, casein (▨) and gelatin (□), was indicated as decrease of A_{570} compared with the negative Mabs 13C7 and 9D5. Mab 9D5* combined with B-ECP of *Aer. salmonicida* subsp. *salmonicida* strain MT004 was used as a positive control

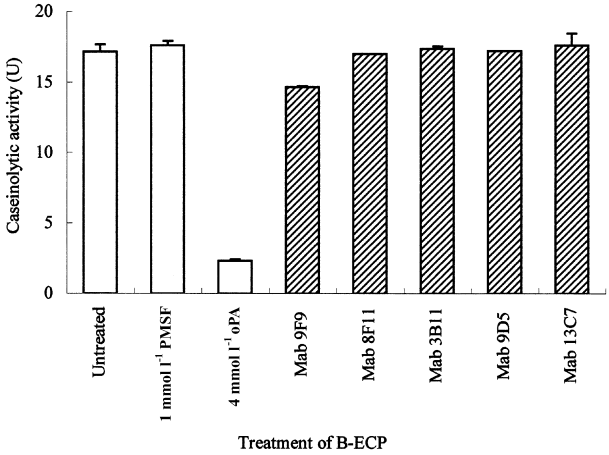

Fig. 4 Inhibitory effect on caseinolytic activity in B-ECP of *Aeromonas salmonicida* subsp. *achromogenes,* strain 265/87, by chemical treatments (□) and monoclonal antibodies (▨). Enzyme activity was measured using azocasein as a substrate. The data shown are mean values ± S.D. of three experiments

activity was found in the concentrated hybridoma supernate itself.

Mab-based ELISA for a serological detection of AsaP1

An indirect ELISA was set up for detecting AsaP1-producing bacterial isolates. Its antigen selectivity was probed using two secondary rabbit antisera, different Mabs, and B-ECPs of several *Aer. salmonicida* strains. As shown in Fig. 5(a), filtrates of six atypical strains, including the type strain NCIMB 1110, gave positive responses with anti-AsaP1-Mabs in combination with a rabbit antiserum against formalin-inactivated O-ECP of the atypical strain 265/87 (Ranti-fECP265/87). Disregarding the higher background signals of 8F11, both Mabs provided comparable results. Isotype-matched control Mabs failed to react under these assay conditions but they indicated the presence of two disparate *Aeromonas* exoproteinases (AspA, P2; Fig. 5b) when employing a rabbit antiserum to the ECP of the typical strain F216·1/83. With this secondary reagent, anti-AsaP1-Mabs showed no reaction.

In a second study comprising a total of 32 strains, the correctness of ELISA data was evaluated by comparing the result of this serological assay with an enzymatic AsaP1 detection. As sample material, ECPs produced by the cellophane overlay method were examined (Table 1). Identical findings were obtained for 24 of 26 atypical strains. By both analyses, AsaP1 was detectable in O-ECP of type strain NCIMB 1110. Two O-ECP preparations were weakly positive by ELISA but graded as AsaP1-negative by enzyme activity studies. Neither enzymatic nor serological examinations provided any indication of AsaP1 production by the typical strains studied.

In order to assess the analytical sensitivity of the AsaP1-ELISA, dilutions of four ECP preparations were subjected to a Mab-based ELISA test and an azocasein assay (Table 2). The serological method proved to be approximately 100-fold more sensitive. AsaP1 was serologically detectable in B-ECP up to dilutions of 1 in 2000 and, in O-ECP probes, at total protein concentrations down to 2 ng ml^{-1}. With the O-ECP preparation of strain 138/88, some relevant quality parameters of the Mab 9F9-based ELISA were estimated more precisely. For this antigen sample, the lower detection limit of the test was determined to be 0·15 ng total protein ml^{-1}. At a protein concentration of 0·74 ng ml^{-1}, the intra- and interassay coefficients of variation (CV) were 3·6% and 6·3%, respectively. In the optimal measuring range of 0·7–20 ng ml^{-1}, the within-run and day-to-day imprecision was $\leqslant$9·2% and $\leqslant$12·3%, respectively.

DISCUSSION

AsaP1 has been shown to be a major exotoxin and a protective antigen of *Aer. salmonicida* subsp. *achromogenes* (Gudmundsdóttir and Magnadóttir 1997). In this paper, the isolation of two Mabs recognizing this toxic exoproteinase is reported. The observed characteristics of the Mabs suggest that they may contribute to studies concerning *in vivo* expression and modes of action of this exotoxin. Moreover, the isolated Mabs may be useful for the screening of DNA libraries for the AsaP1 gene. In the present study, the ELISA application of these antibodies to screen *Aer. salmonicida* isolates for AsaP1 production was examined.

The Mabs were primarily selected according to their capacity for capturing biotinylated ECP-antigens. By this screening technique, two desired hybridomas were isolated. This rather low frequency of specific cell clones might be the result of a considerable molecular modification of AsaP1 by biotin labelling, a weak immunogenicity of this small protein in mice, or its immunomodulatory features (Gudmundsdóttir *et al.* 1995; Gudmundsdóttir and Gudmundsdóttir 1996). Furthermore, the possibility of reduction of the isolated enzyme during handling has to be considered.

The *Aer. salmonicida* subsp. *achromogenes,* strain 265/87, used has been shown to secrete a number of extracellular antigens, including several proteolytic activities (Gudmundsdóttir 1996). Immunoblotting analyses, the recognition of Mab-precipitated 20 kDa antigen by an antiserum against AsaP1, and the measurable caseinolytic but non-detectable gelatinolytic activity of the captured antigen, provided evidence for the AsaP1 specificity of the isolated Mabs. Binding to other tested antigens of *Aer. salmonicida*, including the A-layer protein, two disparate exoproteinases (AspA, P2; Fig. 5b) or LPS, when immobilized on ELISA plates via

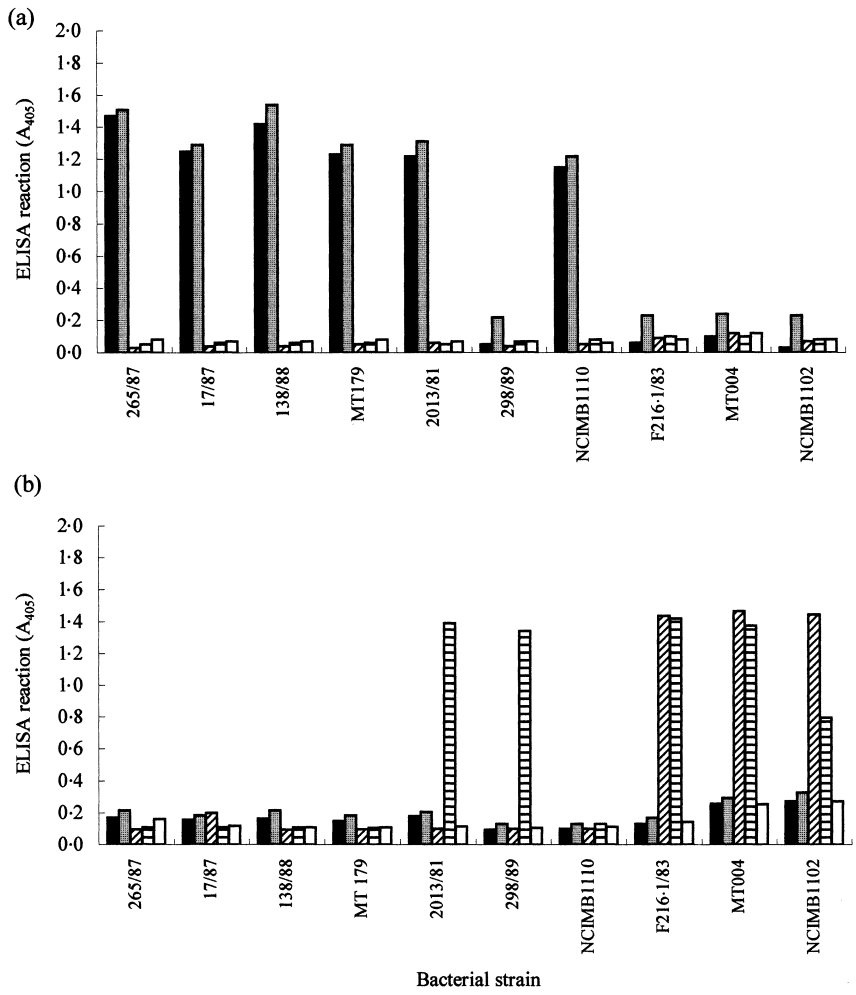

Fig. 5 Specificity analyses of the Mab-based ELISA for detection of AsaP1 in B-ECPs of different *Aeromonas salmonicida* strains. Anti-AsaP1-Mabs, 9F9 (■) and 8F11 (▩), were used for antigen trapping and a rabbit antiserum raised (a) against O-ECP of an atypical strain, 265/87 (Ranti-fECP265/87), or (b) against B-ECP of a typical strain, F216·1/83, as a secondary ELISA reagent. To control the reaction specificities, the negative Mab 13C7 (□), and Mabs 9D5 (▨) and 3B11 (▤), reactive to exoproteinases AspA and P2 of a typical strain, F216·1/83, were employed

polymyxin (Hädge *et al.* 1997) (results not shown), were not observed.

The isolated antibodies are directed against epitopes exposed on the native antigen and therefore, should be suitable for immunoaffinity techniques. As both reacted to denaturation (SDS)-resistant epitopes on immunoblots, it is possible to suggest a recognition of continuous (sequence-dependent) antigenic determinants. The binding of both antibodies to the corresponding antigen of 11 atypical strains verified the recognition of common AsaP1 determinants. Differences between the two Mabs binding abilities were observed. Thus, Mab 9F9 reacted more strongly to the denatured antigen on Western blots, while 8F11 resulted in higher signals in the ECP-capture assay and showed higher background values in the ELISA test. These effects seem to be due to the recognition of different epitopes. The distinct inhibition effects on proteolytic activity indicate different antigenic determinants, where the Mab 9F9-defined epitope is most probably located closer to the active site of the enzyme. However, attempts to discriminate the Mab-defined exotoxin epitopes further by antibody combination and competition studies failed (data not shown).

An interesting characteristic of Mab 9F9 is its negative influence on the caseinase activity in the ECPs. The observed effect appears to be a specific feature of Mab 9F9 because comparable preparations of four other Mabs did not show any influence. In similar experiments with partially purified immunoglobulins from ascites fluids, these results were confirmed (data not shown). In the present study, it was not possible to evaluate the precise extent of AsaP1 inhibition by Mab 9F9, because strain 265/87 produced more than one PMSF-resistant caseinase (Gudmundsdóttir 1996). However, similar reaction intensities of both Mabs in the proteinase-capture assay argued against a very potent specific inhibition.

An indirect ELISA was set up and tested for serological identification of AsaP1-secreting atypical *Aer. salmonicida* strains. Substitutions of immunoreactants revealed that this

Table 2 Evaluation of the Mab-based ELISA in comparison with an enzymatic AsaP1 detection by an azocasein assay. Mab 9F9 was selected as AsaP1-specific antibody and the irrelevant Mab 13C7 was employed to control non-specific sample reaction

Bacterial strain	ECP preparation	ELISA reactivity*		Azocasein assay (U)†
		Mab 9F9	Mab 13C7	
265/87	B-ECP, 1 in 2	1·392	0·068	20·0
	1 in 20	1·391	0·064	2·7
	1 in 200	1·296	0·053	0·3
	1 in 2000	0·593	0·054	0·2
2013/81	B-ECP, 1 in 2	1·295	0·065	1·5
	1 in 20	0·623	0·053	0·4
	1 in 200	0·091	0·051	0·0
138/88	O-ECP, 2000 ng ml^{-1}	1·393	0·069	20·8
	200 ng ml^{-1}	1·362	0·072	3·6
	20 ng ml^{-1}	1·232	0·060	0·3
	2 ng ml^{-1}	0·465	0·064	0·4
MT 179	O-ECP, 2000 ng ml^{-1}	1·376	0·063	1·5
	200 ng ml^{-1}	1·120	0·070	0·2
	20 ng ml^{-1}	0·271	0·074	0·0
	2 ng ml^{-1}	0·063	0·058	0·1

All data represent mean values of two experiments.
* ELISA reactivities of tested sample dilutions were expressed as A_{405} after 60 min development. The cut-off value for Mab 9F9 and Mab 13C7 was 0·08 and 0·10, respectively.
† Sample dilutions were treated with 2 mmol l^{-1} PMSF for 10 min prior to analyses. The cut-off value was considered to be 0·5 U.

assay enabled a specific immunological differentiation of the AsaP1 exotoxin from other, antigenically distinct *Aer. salmonicida* exoproteinases. The possibility of such an unequivocal and effective proteinase differentation is important, as the presence of the AsaP1 enzyme in the ECPs of many atypical strains correlated with lethal and severe pathogenic effects (Gunnlaugsdóttir and Gudmundsdóttir 1997). The relative specificity and sensitivity of the ELISA test was corroborated by examining O-ECPs of 32 *Aer. salmonicida* isolates. By the immunological method, AsaP1 could be identified in the ECPs of 11 of the 26 atypical strains tested, including the type strain for subsp. *achromogenes* (NCIMB 1110). This is in accordance with previous findings (Gudmundsdóttir 1996). When the results of the ELISA test were compared with enzymatic AsaP1 identification, no false-negative serological results were obtained (100% relative sensitivity). On the other hand, two of 23 bacterial isolates graded as AsaP1-negative by enzymatic tests were weakly positive in the ELISA, yielding a relative assay specificity of 91%. Comparison between ELISA and a standard caseinase assay showed that the Mab-based assay was more sensitive. Therefore, it is most probable that the concentration of the AsaP1 antigen in O-ECPs of isolates number 2 and 87:1147 was below the detection limit of the enzymatic methods. However, immunodetection of inactive proteinase or degradation products has to be considered.

The presence of a component that is related to the extracellular metallo-proteinase, P2, of typical strains was detected in the ECPs of two atypical strains (2013/81 and 298/89). This is in accordance with previous data obtained by enzymatic analyses of *Aer. salmonicida* exoproteinases (Gudmundsdóttir 1996). The immunological cross-reaction of this ECP antigen shared by typical and atypical *Aer. salmonicida* strains has not previously been described.

In the ECP of strain 138/88 used for the assessment of some immunoassay quality parameters, the percentage of the AsaP1 antigen was estimated to be approximately 80% of the total protein content (data not shown). As specific assay signals were recorded down to total protein concentrations of 0·15 ng ml^{-1}, the ELISA detection limit for AsaP1 should be approximately 0·12 ng ml^{-1}. Thus, the established serological test provides a useful alternative to previous enzymatic AsaP1 determination and permits a highly sensitive, specific and reliable identification of *Aer. salmonicida* strains expressing this exotoxin *in vitro*. The fundamental parameters of the AsaP1-ELISA were comparable with those of numerous other enzyme immunoassays (Birkeland *et al.* 1985; Brandt *et al.* 1992; Paemen *et al.* 1995), commending its use for evaluation of the exoproteinase antigen in clinical specimens. Moreover, the isolated Mabs and their applicability for ELISA offer a promising approach for the establishment of a specific and sensitive test for monitoring protective humoral immune response in vaccinated fish.

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