Characterization of the receptor-destroying enzyme activity from infectious salmon anaemia virus

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Infectious salmon anaemia virus (ISAV) infects cells via the endocytic pathway and, like many other enveloped viruses, ISAV contains a receptor-destroying enzyme. We have analysed this acetylesterase activity with respect to substrate specificity, enzyme kinetics, inhibitors, temperature and pH stability. The ISAV acetylesterase was inhibited by di-isopropyl fluorophosphate (DFP) in a dose-dependent fashion but not by other known hydrolase inhibitors, suggesting that a serine residue is part of the active site. The pH optimum of the enzyme was in the range 7.5–8.0 and the enzymatic activity was lessened at temperatures above 40°C. The effect of DFP on agglutination/elution of erythrocytes by ISAV demonstrated that the acetylesterase activity is the bona fide receptor-destroying enzyme. A haemadsorption assay was used to analyse whether the esterase was active on the surface of infected cells or not.

Infectious salmon anaemia virus (ISAV) infects cells of farmed Atlantic salmon, Salmo salar L. It has been diagnosed in Norway (Thorud & Djupvik, 1988), Canada (Mullins et al., 1998) and Scotland (Rodger et al., 1998). In addition, ISAV has been shown to replicate in brown trout (Salmo trutta L.) (Nylund et al., 1995), rainbow trout (Oncorhynchus mykiss, Walbaum) (Nylund et al., 1997) and herring (Clupea harengus) (Krosoy et al., 1999) without causing disease. It has also been suggested that ISAV may be transmitted by sea lice (Caligus elongatus and Lepophtheirus salmonis) (Nylund et al., 1993), although it is not known whether ISAV replicates in the lice. Severe anaemia and high mortalities characterize ISAV-infected salmon.

The ISAV genome consists of eight single-stranded RNA segments with negative polarity, suggesting that the virus is orthomyxovirus-like (Mjaaland et al., 1997). Comparison of the conserved motifs of the polymerase gene and the 5′ mRNA ends with corresponding regions of other viruses with a segmented and negative-sense RNA genome shows a closer relationship with members of the Orthomyxoviridae (Krosoy et al., 1999; Sandvik et al., 2000). Recently, the complete sequence and genome organization of a Canadian ISAV isolate was reported (Clouthier et al., 2002). Other known members of this family are the influenza viruses, the Thogoto virus and the Dhori virus. The initial step of influenza infection is binding of the virus particles via their haemagglutinin (HA) to cell-surface sialic acids, and it has recently been demonstrated that the binding of ISAV to the cell surface is neuraminidase-sensitive (Eliassen et al., 2000). Many viruses express enzymatic activity on the surface, for example, the well-known virus sialidases present in influenza A and B, as well as in several paramyxoviruses. Sialidases remove sialic acids present on glycoproteins or glycolipids and are designated RDEs (receptor-destroying enzymes). RDEs of influenza C, several coronaviruses and bovine toroviruses exhibit sialate O-acetylatederase activity as well as receptor-binding activity and are called haemagglutinin esterases (HEs). It has been demonstrated that RDEs...
are required to keep the viruses free of receptor determinants, which would result in the formation of virus aggregates (Hofling et al., 1996). The enzyme has also been suggested to play a role in initiation of infection, either by promoting fusion activity (Huang et al., 1980, 1985) or by releasing sialic acids from oligosaccharides that may interfere with the binding to cellular receptors near the receptor-binding site (Ohuchi et al., 1995). The HEF protein of influenza C virus is known to bind cellular receptors containing 9-O-acetyl-5-N-acetylsialic acids as the major receptor determinant and releases the acetyl residue from position C-9 of the substrate (Herrler & Klenk, 1987; Rogers et al., 1986). ISAV has also been shown to possess acetylemsterase activity, and no neuraminidase activity has been detected, suggesting the acetylemesterase as the putative RDE of ISAV. The virus is able to agglutinate and spontaneously elute from erythrocytes in several fish species, except Atlantic salmon erythrocytes, which remain agglutinated. Further studies have suggested that ISAV uses an unknown receptor determinant different from that used by influenza A, B and C viruses (Falk et al., 1997). In the present study, further characterization of the acetylemsterase activity of ISAV is presented. Several synthetic and natural inhibitors have been tested to examine the enzyme activity.

Esterases can be classified according to their inhibition profiles. We therefore tested the effect of various inhibitors and possible competing natural substrates (all obtained from Sigma Aldrich) on the ISAV esterase (strain Glesvaer 2/90). Virus was cultured in a cell line established from Atlantic head kidney cells (SHK-1 cells) (Dannevig et al., 1995). Twenty-five µg of purified virus was incubated with 1 mM p-nitrophenyl acetate (pNPA) and assayed at 405 nm. Alternatively, enzyme activity was assayed with the fluorogenic substrate 4-methylumbelliferyl acetate (4-MUAc). From Table 1 it can be seen that only di-isopropyl fluorophosphate (DFP) was an effective inhibitor of this enzyme. None of the glycoconjugates (with or without O-acetyl groups) tested had any effect on the rate of hydrolysis. Falk et al. (1997) have shown that the ISAV esterase has a substrate specificity different from influenza C virus HEF and the bovine coronavirus acetylemesterase (Herrler et al., 1985; Rogers et al., 1986; Vlasak et al., 1987). However, several synthetic compounds with acetate as the leaving group, e.g. pNPA, 4MUAc and α-naphthyl acetate are all cleaved by these esterases (Garcia-Sastre et al., 1991; Vlasak et al., 1987; Zimmer et al., 1992). We tested substrates with propionate or phosphate as the leaving groups but these were not hydrolysed by the ISAV esterase (not shown). PMSF is normally used as a substitute for the extremely toxic DFP in inhibition studies, but we could not detect any inhibition with this compound. It has been shown that 1 mM DFP completely inactivates the influenza C virus esterase, but PMSF was less effective, giving only an 8% inhibition at a 1 mM concentration (Muchmore & Varki, 1987). It has also been shown that the influenza C virus esterase activity could be slightly inhibited using 10 mM physostigmine (25% inhibition) (Schauer et al., 1988). All of these compounds attack a serine in the active site of the enzyme, and PMSF seems to inhibit a restrictive range of serine proteases such as trypsin, chymotrypsin and mammalian acetylcholinesterase (Moss & Fahney, 1978; Turini et al., 1969). The Michaelis constant, $K_m$, was determined with the fluorogenic substrate 4-MUAc in the range 1–500 µM and

![Fig. 1. Michaelis–Menten kinetics and double reciprocal plot for hydrolysis of 4-methylumbelliferyl acetate by 25 µg/ml ISAV protein at 20 °C, pH 7.4.](image-url)
Receptor destroying activity in ISAV

Fig. 2. Effect of temperature (A) and pH (B) on enzymatic activity. (A) Samples of 25 µg ISAV protein were incubated for 1 h at different temperatures before assaying the acetylesterase activity with 200 µM 4-MUAc. (B) Samples of 25 µg ISAV protein were incubated with 1 mM pNPA diluted in PBS at different pHs to determine the optimal pH for the acetylesterase activity. The pH range used was pH 5–8–8–0.

Fig. 3. Acetylesterase activity in infected SHK-1 cells (A) and medium (B). SHK-1 cells were grown in six-well tissue culture plates, and duplicate wells were infected with ISAV. At various time intervals, esterase activity was assayed in cells and medium.

found to be about 75 µM at pH 7–4 (Fig. 1). Compared with other viral acetylesterases, the ISAV esterase metabolized 4-MUAc with intermediate efficiency ($K_m = 75$ µM). In a comparative study by Wurzer et al. (2002), the $K_m$ for three types of coronavirus esterases was found to be in the range 20–94 µM. The corresponding value for the influenza C virus esterase is 210 µM. The temperature stability of the enzyme was tested by incubation of ISAV at different temperatures for 1 h before analysis of the esterase activity. The enzyme remained stable up to about 40 °C, but the activity fell sharply when exposed to higher temperatures (Fig. 2A). Above 40 °C, the acetylesterase was largely inactivated after 60 min of incubation. Similar characteristics have been found for the influenza C virus (C/Johannesburg/1/66) esterase (Garcia-Sastre et al., 1991). Most enzymes have a characteristic pH at which the catalytic reaction is maximal. To determine the pH sensitivity of the ISAV esterase, virus was diluted in buffers at different pHs and incubated with 1 mM substrate (pNPA). Because of substrate autohydrolysis at high temperature or high pH, it was difficult to measure specific esterase hydrolysis at pH > 8–0. From Fig. 2(B), it can be seen that the ISAV acetylesterase expressed its maximum activity in the range pH 7–5–8–0. To examine the kinetics of esterase expression on the surface of infected SHK-1 cells and on secreted viral particles, the surface and medium activity were measured at different time intervals after infection with ISAV. Activity was analysed directly in 30 mm culture dishes with a confluent SHK-1 cell layer. Medium was transferred to centrifuge tubes and viral particles pelleted at 100 000 g, whereas the remaining cells were washed with PBS and assayed directly in a Perkin
Elmer HTS 700 plate reader. Non-infected SHK-1 cells served as a control. On the cell surface, activity was increased 7–8 days after infection (Fig. 3A). Concomitant with surface expression, esterase activity in the medium (sedimentable at 100000 g) also increased from day 7 onward, indicating budding of virus with active esterase (Fig. 3B).

To determine whether the acetyl esterase activity we analysed was the receptor-destroying enzyme (RDE), we compared the effect of DFP on haemagglutination and elution of red blood cells (RBCs) from salmon, trout and five mammals (human, cow, pig, sheep and horse). Agglutination induced by ISAV was observed for RBCs from trout, salmon and horse, whereas the others were negative. Bovine submaxillary mucin could prevent agglutination of salmon, trout and horse RBCs. When the agglutinated cells were further incubated for 2 h, the cells from trout and horse were eluted, whereas the salmon cells remained agglutinated. When ISAV was preincubated with 1 mM DFP, complete inhibition of elution was observed for trout and horse RBCs (results not shown). This shows that the acetyl esterase represents the bona fide RDE of ISAV and supports the former results, which claim that an RDE is needed for release of newly synthesized virus particles. ISAV was able to agglutinate erythrocytes from at least one mammalian species (horse) and several fish species, including Atlantic salmon. Horse and cow erythrocytes contain mainly N-glycolyl sialic acids (100% for horses and 93% for cows) (Suzuki et al., 1992) and those from humans contain only N-acetylated sialic acids. Human erythrocytes contain both sialic acids (SA) with $\alpha_2,3$Gal and $\alpha_2,6$Gal linked to the penultimate galactose residue ($SA\alpha_2,3Gal$ and $SA\alpha_2,6Gal$). In contrast, horse and cow erythrocytes contain mainly the $SA\alpha_2,3Gal$ linkage. By agglutination studies, we have shown that the acetyl esterase activity is in fact the RDE of ISAV, and that the virus was able to agglutinate mammalian erythrocytes, which contain only N-glycolyl neuraminic acid. These results demonstrate that the ISAV esterase shares several characteristics with the influenza C virus esterase (acetyl as the leaving group, inhibition by DFP, $K_{\text{in}}$, pH and temperature sensitivity), but there are also important differences (low inhibition by PMSF and eserine). In addition, reciprocal reagglutination with ISAV and influenza C virus after elution have demonstrated that these viruses do not destroy each other’s receptors (Falk et al., 1997). Detailed knowledge of the RDE activity in pathogenic viruses may lead to development of therapeutic strategies such as the neuraminidase inhibitors used to treat influenza A virus infections in humans. In parallel with vaccine development, more detailed studies of putative ISAV RDE inhibitors may also be a pathway leading to more efficient control of this pathogenic virus.

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References


Infectious salmon anemia virus (ISAV) in brown trout. *Journal of Aquatic Animal Health* 7, 236–240.


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