Inosine-arginine salt as a promising agent for *in vitro* activation of waterborne actinospores of fish pathogenic myxozoans

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Running header: Actinospore activation by ino.arg salt

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Abstract

Since the recent finding that mucus-derived nucleosides serve as the key host cues for myxozoan actinospore fish host recognition, their use for experimental actinospore activation in the laboratory or application in disease prevention has not progressed yet. One obstacle has been the low solubility of pure inosine and guanosine. To overcome this, we used inosine-arginine salt, which was found to incorporate both high activation properties and high solubility. We tested its efficacy both in microassays directly observing reactions of actinospores of two distantly related myxozoan species, *Myxobolus cerebralis* and *Myxobolus pseudodispar* in comparison to inosine, as well as its actinospore-inactivation properties by preliminary polar capsule discharge in an infection experiment. The substance was considerably more effective in eliciting polar capsule discharge and sporoplasm emission at much lower concentrations than pure inosine and, in contrast to the latter, remained dissolved in aqueous solution. Ionsine-arginine exposure of *M. pseudodispar* actinospores apparently resulted in polar capsule discharge and sporoplasm emission before host contact and subsequently in a lower infection rate in roach, *Rutilus rutilus*.

Introduction

Myxozoan parasite transmission to teleost fish hosts ultimately depends on their spread by actinospore stages. These planktonic stages are shed from their invertebrate host, mainly oligochaete worms, and encounter their fish hosts by passive floating and unique reactions in close vicinity of a suitable host. A combination of chemical and mechanical signals initiates the invasion process (Yokoyama et al. 1995, Kallert et al. 2005, Eszterbauer et al. 2009). For actinospores of several species (e.g. *Myxobolus cerebralis, M. pseudodispar, M. parviformis, Henneguya nuesslini*), chemical recognition of nucleosides (i.e. inosine and guanosine) prime the actinospore to become mechanically sensitive (Kallert et al. 2010, Kallert et al. 2011). Hitherto, nucleosides are the only natural host cues found in fish mucus to elicit distinct reactions by actinospores. When contact occurs, upon a mechanical stimulus they anchor themselves to the host surface by extrusion of their polar filaments, threads that are wound up in the myxozoan-specific polar capsules. These pull the endospore unit closely to the epidermal surface and enable subsequent penetration of the amoeboid sporoplasm primary cell that thereafter enters the tissue and further migrates towards deeper tissue layers for secondary cell release (Kallert et al. 2009).

An easy-to-use *in vitro* activation of actinospores would not only enable researchers to test for actinospore reactivity and infectivity, but is an important step to analyse genetic factors involved in spore activation and host invasion as well as host specificity parameters (Eszterbauer et al. 2009). It also has to be a first step for *in vitro* culturing of myxozoans. The identification of the natural stimulants for actinospore activation initially opened the door for such study possibilities. However, inosine and guanosine, intermediate metabolites of ATP breakdown and the key components recognized by actinospore stages, have relatively low water solubility and because of their hydrophobic character tend to disappear from the water column rapidly to form either molecular aggregates or bind to various substrates. While this

may be one key characteristic for serving as a suitable host signal (accumulation in the mucus and virtual absence in the water column), these compounds use in scientific studies and application in aquatic environments remains rather difficult. Solubilisation requires heating and constant stirring; otherwise these substances attach to debris, glass walls or build aforementioned aggregates. Substitution by other, more soluble nucleoside derivates was not or less effective as compared to the pure substances in preliminary tests (unpublished data, own experiments). Besides, these substitutes are usually very costly. An alternative might be inosine-arginine (ino.arg) salt patented by Kurauchi et al. (2004), a substance which has a high water solubility even at low temperatures. It has been developed for crop nutrition and growth promotion and is comparatively cheap in production. Therefore it seemed to be a suitable means for application in actinospore activation.

Here we present the evaluation of the efficacy of the ino.arg salt in laboratory usage for polar capsule discharge triggering and sporoplasm emission tested with two myxosporean actinospores. For the study, we chose *M. cerebralis*, the parasite causing whirling disease in salmonids, and *M. pseudodispar*, a widely distributed muscular parasite of roach and other cyprinids throughout Europe. Furthermore, we asked whether this chemical may be a potent preliminary activator of actinospores in absence of fish to reduce numbers of infective stages in water and subsequently to quantitatively interfere with transmission to host fish reducing disease severity.

Material & Methods

Parasites

Actinospores aged less than 48 hrs of *M. cerebralis* and *M. pseudodispar* were obtained from long term laboratory cycles as described previously (Kallert et al. 2005, 2007). They were filtered by 20 µm nylon gauze from the supernatant water of mud bottom tanks containing oligochaete worms infected with the parasites, and they were further concentrated by gauze

filtration for experimental use and kept at 12°C at all times. The concentration of actinospores used or microassays varied between 2,500 and 20,000 per ml. The same actinospore isolate was used for each microassay replicate.

Fish and mucus preparation

SPF roach, *Rutilus rutilus*, were laboratory-reared from eggs collected from a small stream near Budapest, Hungary. and used for infection at 1.2 cm (5 months of age). They were kept in aerated glass tanks at 23°C and fed on commercial ornamental fish food. Rainbow trout for mucus homogenate preparation (positive controls) were obtained live from a commercial fish farm in Germany. Mucus homogenate was prepared as described previously by Kallert et al (2005). The concentration of mucus was calculated based on its dry weight after complete lyophilisation of 200 µl aliquots. A homogenate of 1 mg ml^{-1} final concentration was used for actinospore activation.

Ino.arg preparation

Ino.arg was prepared according to the patent instructions (US patent no. 2004/0192553 A1 (Kurauchi et al. 2004). Briefly, 10 g of inosine (Sigma, pure grade) and 6.5 g of arginine (Sigma, HPLC grade) (equimolar amounts) were solubilized by stirring and heating (70°C) to obtain a concentrated solution in 33.5 ml water. This was slowly added to 1 l of anhydrous ethanol under constant vigorous stirring. The resulting white precipitate was paper filtered (round paper filters, Scholl) and dried at 45°C overnight. The white powder of ino.arg was then diluted with 5 mM sodium phosphate buffer pH 7.5 to concentrations of 0.1, 0.05, and 0.01 mg ml^{-1}, respectively.

Experiments

Activation microassay

106 To measure polar filament discharge and sporoplasm emission, we applied the vibration
107 microassay including trout mucus homogenate as described previously (Kallert et al. 2005).
108 The actinospore solution of *M. cerebralis* and *M. pseudodispar* was mixed with test substrate
109 at a ratio of 2.33:1 (total volume 30 µl) on a slide and vibrated (50 Hz, 3 mm amplitude)
110 immediately after covering with a 22×22 mm cover slip. Activated actinospores were then
111 counted using phase contrast microscopy for approximately 5 min immediately after
112 activation. Reaction rates were calculated from all viable actinospores per slide with clear
113 reactions or their absence. Discharge and emission were always counted as separate reactions,
114 one spore is either just discharged or has emitted its sporoplasm. We did not count
115 actinospores with emitted sporoplasms that were not close or attached to the apical region or
116 the ones without sporoplasms. Only sporoplasms inside the actinospore shell which reacted to
117 stimuli or the emitted sporoplasms in close contact were counted (see e.g. Fig. 1 by
118 Eszterbauer et al. 2009). All test substrates were buffered to pH 7.5 by sodium phosphate
119 buffer. As negative control substrate, aqueous solutions of the non-stimulating nucleoside
120 derivate 3´methyl-guanosine were used with the final concentration of 0.1 and 1 mg ml^{-1}. For
121 the inosine substrate, inosine powder (Sigma) was diluted with 5 mM sodium phosphate
122 buffer (pH 7.5) to concentrations 0.5 and 0.1 mg ml^{-1}, respectively. Preparation of substrates
123 was conducted in respect of a degree of inosine solubilisation comparable to its use in a field-
124 like situation by leaving samples standing after preparation and buffering at 12°C for 20 min
125 with only minimum vortexing prior to addition to the actinospore suspension. These
126 conditions were chosen to more closely resemble the practical application of inosine and
127 ino.arg salt in our laboratory setup.
128 In the first activation microassay, substrate concentrations were chosen on the basis of
129 preliminary tests, the experience gained from previous experiments and in accordance to the
130 inosine content of trout mucus as published by Kallert et al. (2011). In a second microassay
131 experiment, we intended to confirm the results from the first experiment with another ino.arg

preparation and a different day`s actinospore isolate and include an even lower (0.01 mg ml^{-1}) ino.arg concentration to highlight the differential efficiency properties.

Decoy-Assay

In this part of the study, we tested the possibility of reduction of infective units (actinospores) prior to individual fish exposure after the incubation of *M. pseudodispar* actinospores in water with ino.arg in comparison to pure inosine and water control without additives. To discover the potential of a reduction of numbers of infective actinospores by ino.arg in bigger water volumes and to lower subsequent fish infection, we treated water (300 ml) containing *M. pseudodispar* actinospores (1000 per glass and single fish) with inosine (0.01 mg l^{-1}), ino.arg (0.018 mg l^{-1}) and tap water (gently stirred with a glass rod twice) as a negative control for 20 min prior to addition of SPF fish (10 specimens per group). Individually exposed fish were left in the aerated solution for 2.5 h before transfer to aerated aquaria. Fish of the same group were kept together. After 3 months, fish were anaesthetized in 200 mg l^{-1} MS222 solution (Sigma) and killed by cranial cut. After dissection, the musculature in one side fillet of each specimen was homogenized with Ultra Turrax, the homogenate was diluted to 7 ml with cooled tap water. The numbers of myxospores were counted in four replicates per homogenate using a Bürker chamber with phase contrast microscopy.

Statistics

To test for normal distribution of data, the Kolmogorov-Smirnov test was used while homogeneity of variances was confirmed by the Levene test. Data from myxospore enumeration in the infection experiment and the activation microassays were compared using ANOVA. Probability levels for reaction rates between all groups were analysed by a multiple t-test (Tukey LSD). For all statistics, SPSS 11.5 and StatistiXL were used.

Results

Activation microassay

In both microassay experiments, dissolved ino.arg salt showed a better activation effect than pure inosine. In all cases, controls were significantly different from mucus homogenate reaction rates ($P < 0.05$). In the first experiment, we observed rather low reaction rates of polar filament discharge (Table 1). Sporoplasm emission rates showed species-specific differences. For *M. pseudodispar*, higher sporoplasm emission rates were detected than for *M. cerebralis*. The sporoplasm emission rate was highest in the mucus homogenate positive control for both species. When directly compared in the microassay, ino.arg could reach higher activation levels than solubilised inosine, especially in the sporoplasm emission response (Table 1), albeit these results could not reach a significant level.

In the second experiment, the activation of both reactions in both species` actinospores by ino.arg was significantly greater ($P < 0.05$) than that elicited by inosine solution of the same concentration. In the case of *M. pseudodispar* polar capsule discharge, the same was observed for the 10-fold lower ino.arg concentration ($P < 0.05$). Ino.arg in a 0.1 mg ml^{-1} concentration outperformed the highly effective substrate prepared from natural trout mucus regarding polar filament discharge by 20% (Table 1, $P < 0.05$ for *M. cerebralis*). Sporoplasm emission was always significantly different ($P < 0.001$) after ino.arg (0.1 mg ml^{-1}) addition when compared to control. In *M. pseudodispar*, polar filament discharge induced by 0.1 mg ml^{-1} ino.arg was also higher, but not significantly different from that by mucus.

Decoy experiment

Roach were heavily infected in the control group (without prior addition of a substrate) but only four specimens survived in this group out of the 10 fish until end of the experiment, while seven survived in the two other groups respectively. The relative mean numbers of myxospores in single homogenized fillets (one side) of infected roach were 181,250 ± 38,500 (negative control), 200,000 ± 88,250 (inosine) and 90,000 ± 43,750

(ino.arg) (myxospore number per ml ± standard error of the mean). Though not significantly different (P = 0.28, t-test), ino.arg decreased parasite load to a mean of 45% of that obtained by pure inosine addition. This result was not statistically significant due to the low final control group size.

Discussion

M. pseudodispar actinospores are more delicate regarding their apical architecture and thus always react much quicker and to a greater extent than the more rigidly built *M. cerebralis* stages (Kallert & El-Matbouli 2008). Therefore, we examined the first 5 mins after the actinospore activation to be able to compare also polar capsule discharge rates, which is not to such a high extent concealed by emerging sporoplasms that accumulate with incubation time. In the first activation microassay, we used relatively high inosine concentration (i.e. 0.5 mg ml^{-1}) compared to the inosine content in a highly effective mucus preparation, so the difference was not as elevated (i.e. not significant), while ino.arg could exceed the reaction level of inosine and reach that of the mucus homogenate (especially for *M. cerebralis*) due to the sufficiently high proportion of dissolved inosine. The sporoplasm emission rate was highest in the mucus homogenate positive control for both species, as sporoplasm emission took place more rapidly, most likely due to slightly higher mucus homogenate viscosity and better mechanical stimulation. Compared to *M. pseudodispar*, *M. cerebralis* showed lower sporoplasm emission rates during the relatively short counting time, which might be explained by their more rigid actinospore architecture.

Inosine solubilisation requires heating, and it has a water solubility of only 2.1 g l^{-1} at 20°C. In cold water, it rapidly forms aggregates due to its slightly hydrophobic nature (log partition factor P = 1.4, Novotny et al. (2000)), which can be readily observed under the microscope. Thereby, it's triggering activity as a host cue decreases rapidly at ambient

temperatures. The chemical formula of ino.arg is $C_{16}H_{26}N_8O_7$, and when complexed with arginine, it dissociates readily in polar solutions. This makes all functional groups of the nucleoside available, since a major conformation change is not expected. This is reflected by the excellent activation efficacy for actinospores that we observed in this study. Since not only polar filament discharge was triggered, but a high proportion of sporoplasm emission was achieved such as it takes place in full mucus preparations, artificial effects (e.g. by discharge due to osmotic or pH-effects) can be excluded. The stimulation thus can be regarded as the natural reaction taking place in vicinity of a fish host prior to invasion. The fact that ino.arg is effective even at much lower concentrations than inosine underlines its homogenous availability to the actinospore receptor(s) in water. Furthermore, it remains solubilised (retains its activating properties) even when left in solution for 4 d, and not losing its triggering effect (data not shown).

Parasite load is an indirect measure of infectivity ratio among waterborne actinospores (Ryce et al. 2005). The more units there are in the water column, the more will be able to enter a fish host in a given time and theoretically more mature myxospores will develop. It is not known whether some of the fish used in the experiment died from overexposure or for other reasons like a secondary infection. However, unfortunately, the differences in the results could not reach sufficient significance levels due to this instance. When actinospores react by polar filament discharge without anchoring to a host, this affects mainly the capsulogenic cell itself. After polar capsule discharge, these cells disintegrate and the underlying sporoplasm may be hampered by osmotic problems. This also instantly occurs, when in addition to discharge the sporoplasm is activated and it actively emerges from the shell valves. After a short period, the sporoplasm stops to move and disintegration due to osmotic imbalance likely takes place. Due to the rapid emergence and subsequent sporoplasm cell death, these specimens will not be infective for fish thereafter. Although not statistically significant due to the few fish used for this challenge and a high variation between individual parasite loads, the

result indicates the potency of ino.arg to act as a potent preliminary polar capsule discharge/sporoplasm emission trigger leaving parts of the parasite load noninfective in the water.

Diseases caused by myxozoans are still a major cause for economic losses in the wild and in aquaculture. Means to remove infective actinospores from waters usually involve UV-irradiation, mechanical filter methods (e.g. sand filtration) or would require the use of water disinfectants, which can be toxic for fish or the environment or are even illegal. An overview on protective measures both in aquacultures and field systems was provided by Steinbach et al. (2009). According to our results, after adequate toxicologial and water and food safety tests, addition of rather low concentrations of ino.arg could possibly be used for at least partial, but effective water clearance of infective actinospores to support disease prevention even at low water temperatures. Furthermore, it can serve as an easy-to-use laboratory standard for actinospore activation for various studies. The latter application could be important for researchers aiming to study myxozoan genetics and the basics of the invasion processes (receptor studies, genetic regulation and cell recognition) and is supposedly a valuable tool for future high-yield *in vitro* culturing or cell culture of myxozoans without the contamination that go along with mucus use.

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289 Tables

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291 **Table 1. (a)** Activation microassay #1 using *Myxobolus cerebralis* and *Myxobolus*
292 *pseudodispar* actinospores (6 replicates), and **(b)** Activation microassay #2 with new ino.arg
293 production lot including lower concentrations and different actinospore isolates of both
294 species (12 replicates). Mean rate (%) of actinospores showing polar filament discharge and
295 sporoplasm emission upon chemical stimulation followed by instant mechanical activation.
296 Ino = Inosine constantly solubilized (final conc. thereafter), IA = inosine.arginine salt, Mucus
297 = trout mucus homogenate (final conc. 1 mg ml^{-1}), Negative control = methyl-guanosine 1 mg
298 ml^{-1}, *N* = actinospores counted, SEM = standard error of the mean. *: significant difference
299 from negative control ($P<0.05$).

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	Polar Filament Discharge	+/- SEM	Sporoplasm-Emission	+/- SEM	*N*
(a) Activation microassay #1					
M. cerebralis					
Negative control	12.12	2.24	0	-	165
Ino 0.5 mg ml^{-1}	27.75	3.65	9.56	3.63	251
IA 0.05 mg ml^{-1}	31.06	4.49	12.31	4.52	268
Mucus	32.56	2.13	14.34	6.21	251
M. pseudodispar					
Negative control	6.92	2.82	0	-	159
Ino 0.5 mg ml^{-1}	11.29	3.15	28.74	8.19	174
IA 0.05 mg ml^{-1}	11.63	1.87	35.82	4.80	201
Mucus	18.03	9.03	51.20	7.0	125
(b) Activation microassay #2					
M. cerebralis					
Negative control	22.50	6.76	0	-	195
Ino 0.1 mg ml^{-1}	36.36	4.38	2.76	1.52	181
IA 0.01 mg ml^{-1}	27.16	4.40	1.62	0.87	243
IA 0.1 mg ml^{-1}	58.27	2.36	3.62	1.33	219
Mucus	38.67	5.08	6.22	1.30	193
M. pseudodispar					
Negative control	7.32	2.68	2.38	1.20	252
Ino 0.1 mg ml^{-1}	11.95	2.50	22.53	3.75	342
IA 0.01 mg ml^{-1}	15.73	3.12	17.08	3.68	267
IA 0.1 mg ml^{-1}	38.64	7.91	30.34	3.86	379
Mucus	34.62	3.84	43.65	5.31	323

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