

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

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10 Running header: Actinospore activation by ino.arg salt

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16

17 **Abstract**

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

32

33 **Introduction**

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

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

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

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

77 **Material & Methods**

78 *Parasites*

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

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

86 *Fish and mucus preparation*

87 SPF roach, *Rutilus rutilus*, were laboratory-reared from eggs collected from a small stream
88 near Budapest, Hungary. and used for infection at 1.2 cm (5 months of age). They were kept
89 in aerated glass tanks at 23°C and fed on commercial ornamental fish food. Rainbow trout for
90 mucus homogenate preparation (positive controls) were obtained live from a commercial fish
91 farm in Germany. Mucus homogenate was prepared as described previously by Kallert et al
92 (2005). The concentration of mucus was calculated based on its dry weight after complete
93 lyophilisation of 200 µl aliquots. A homogenate of 1 mg ml⁻¹ final concentration was used for
94 actinospore activation.

95 *Ino.arg preparation*

96 Ino.arg was prepared according to the patent instructions (US patent no. 2004/0192553 A1
97 (Kurauchi et al. 2004). Briefly, 10 g of inosine (Sigma, pure grade) and 6.5 g of arginine
98 (Sigma, HPLC grade) (equimolar amounts) were solubilized by stirring and heating (70°C) to
99 obtain a concentrated solution in 33.5 ml water. This was slowly added to 1 l of anhydrous
100 ethanol under constant vigorous stirring. The resulting white precipitate was paper filtered
101 (round paper filters, Scholl) and dried at 45°C overnight. The white powder of ino.arg was
102 then diluted with 5 mM sodium phosphate buffer pH 7.5 to concentrations of 0.1, 0.05, and
103 0.01 mg ml⁻¹, respectively.

104 *Experiments*

105 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 μ l) on a slide and vibrated (50 Hz, 3 mm amplitude)
110 immediately after covering with a 22 \times 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

132 preparation and a different day's actinospore isolate and include an even lower (0.01 mg ml⁻¹)
133 ino.arg concentration to highlight the differential efficiency properties.

134 Decoy-Assay

135 In this part of the study, we tested the possibility of reduction of infective units (actinospores)
136 prior to individual fish exposure after the incubation of *M. pseudodispar* actinospores in water
137 with ino.arg in comparison to pure inosine and water control without additives. To discover
138 the potential of a reduction of numbers of infective actinospores by ino.arg in bigger water
139 volumes and to lower subsequent fish infection, we treated water (300 ml) containing *M.*
140 *pseudodispar* actinospores (1000 per glass and single fish) with inosine (0.01 mg l⁻¹), ino.arg
141 (0.018 mg l⁻¹) and tap water (gently stirred with a glass rod twice) as a negative control for 20
142 min prior to addition of SPF fish (10 specimens per group). Individually exposed fish were
143 left in the aerated solution for 2.5 h before transfer to aerated aquaria. Fish of the same group
144 were kept together. After 3 months, fish were anaesthetized in 200 mg l⁻¹ MS222 solution
145 (Sigma) and killed by cranial cut. After dissection, the musculature in one side fillet of each
146 specimen was homogenized with Ultra Turrax, the homogenate was diluted to 7 ml with
147 cooled tap water. The numbers of myxospores were counted in four replicates per
148 homogenate using a Bürker chamber with phase contrast microscopy.

149 *Statistics*

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

155 **Results**

156 *Activation microassay*

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

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

175

176 *Decoy experiment*

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

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

186

187 **Discussion**

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

203 Inosine solubilisation requires heating, and it has a water solubility of only 2.1 g l⁻¹ at
204 20°C. In cold water, it rapidly forms aggregates due to its slightly hydrophobic nature (log
205 partition factor $P = 1.4$, Novotny et al. (2000)), which can be readily observed under the
206 microscope. Thereby, its triggering activity as a host cue decreases rapidly at ambient

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

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

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

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

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254

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288

289 Tables

290

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).

300

	Polar Filament Discharge	+/- SEM	Sporoplasm-Emission	+/- SEM	<i>N</i>
(a) Activation microassay #1					
<i>M. cerebralis</i>					
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
<i>M. pseudodispar</i>					
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					
<i>M. cerebralis</i>					
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
<i>M. pseudodispar</i>					
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

