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## **Chicken Lung Lectin is a functional C-type lectin and inhibits haemagglutination by Influenza A Virus**

Astrid Hogenkamp, Najiha Isohadouten, Sylvia S.N. Reemers, Roland A. Romijn, Wieger Hemrika, Mitchell R. White, Boris Tefsen, Lonneke Vervelde, Martin van Eijk, Edwin J.A. Veldhuizen, et al.

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1 Chicken Lung Lectin is a functional C-type lectin and inhibits haemagglutination by  
2 Influenza A Virus

3

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23 **Abstract**

24

25 Many proteins of the calcium-dependent (C-type) lectin family have been shown to  
26 play an important role in innate immunity. They can bind to a broad range of  
27 carbohydrates, which enables them to interact with ligands present on the surface of  
28 micro-organisms. We previously reported the finding of a new putative chicken  
29 lectin, which was predominantly localized to the respiratory tract, and thus termed  
30 chicken Lung Lectin (cLL). In order to investigate the biochemical and biophysical  
31 properties of cLL, the recombinant protein was expressed, affinity purified and  
32 characterized. Recombinant cLL was expressed as four differently sized peptides,  
33 which is most likely due to post-translational modification. Crosslinking of the  
34 protein led to the formation of two high-molecular weight products, indicating that  
35 cLL forms trimeric and possibly even multimeric subunits. cLL was shown to have  
36 lectin activity, preferentially binding to  $\alpha$ -mannose in a calcium-dependent manner.  
37 Furthermore, cLL was shown to inhibit the haemagglutination-activity of human  
38 isolates of Influenza A Virus, subtype H3N2 and H1N1. These result how that cLL is  
39 a true C-type lectin with a very specific sugar specificity, and that this chicken lectin  
40 could play an important role in innate immunity.

41

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43

44

45 Key words: Chicken Lung Lectin; Innate Immunity; Influenza A Virus; C-type

46 Lectin

## 47 **1. Introduction**

48

49 The innate immune system provides a first line defense against potential pathogens,  
50 bridging the interval between exposure to the pathogen and the specific response of  
51 the adaptive immune system. The effectiveness of the innate immune system highly  
52 depends on the recognition of pathogens. For this purpose, the innate immune system  
53 relies on pattern recognition molecules which are capable of binding to regular  
54 patterns of carbohydrates present on the surface of pathogens. Within this group of  
55 pattern recognition molecules, the calcium-dependent (C-type) lectins represent a  
56 family of proteins which are found throughout the animal kingdom (Day, 1994).  
57 These proteins share a structural homology in their carbohydrate recognition  
58 domains (CRD) but differ with respect to their carbohydrate specificity. We recently  
59 reported the discovery of a chicken lectin which was designated chicken Lung Lectin  
60 (cLL) due to its predominant expression in the chicken respiratory system  
61 (Hogenkamp et al., 2006). Based on sequence homology, cLL was identified as a C-  
62 type lectin, and although it could not be classified as a collectin due to its lack of a  
63 collagen domain, the sequence of its CRD was found to be most homologous to that  
64 of the collectin Surfactant Protein A (SP-A) (39% - 45% similarity, depending on the  
65 species, (Hogenkamp et al., 2006). To our knowledge, no other C-type lectins with  
66 more structural similarities to cLL have been identified to date. Collectins and  
67 several other C-type lectins have been identified as important molecules in innate  
68 immunity. For example, SP-A plays an important role in innate defense against  
69 invading bacterial pathogens in the lung (reviewed in Wright et al., 2001 and  
70 Hogenkamp et al., 2007), as well as viruses, including Influenza A Virus (IAV).  
71 Other lectins such as RegIIIγ (Narushima et al., 1997), and its human homologue

72 HIP/PAP (Lasserre et al., 1992), affect the gut flora composition and have been  
73 shown to possess direct antimicrobial properties (Cash et al., 2006). Tetranectin is a  
74 secreted C-type lectin (Berglund and Petersen, 1992; Sørensen et al., 1995) which  
75 has also been found in chicken (unpublished data, GenBank accession no.  
76 AJ277116). Its function is not quite clear yet, but it has been suggested to play a role  
77 in cellular immunity (Stoevring et al., 2005). In this study, we expressed recombinant  
78 cLL to investigate the structural and functional characteristics of this protein. The  
79 carbohydrate-specificity of cLL was tested, and the putative protective role of cLL  
80 was studied by investigating haemagglutination-inhibition and neutralization of viral  
81 infectivity of various IAV strains.

## 82 **2. Materials and Methods**

83

### 84 *2.1. RNA extraction and cDNA synthesis*

85 Total cellular RNA from lung tissue from healthy female Ross 308 broiler chicken  
86 was extracted using TRIzol© (Invitrogen, Carlsbad, CA) and Magnalyser Green  
87 Beads (Roche Diagnostics GmbH, Mannheim, Germany Diagnostics GmbH,  
88 Mannheim, Germany). The cDNAs used as templates in PCR were synthesized using  
89 1 µg DNase I treated RNA with M-MLV-RT and 500 µg/ml oligo dT12-18 primers  
90 (Invitrogen, Carlsbad, CA) in a 20 µl reaction volume with incubation at 37°C for 50  
91 min.

92

### 93 *2.2. Polymerase chain reaction and amplified DNA fragment isolation*

94 To amplify cDNA of the mature peptide sequence of cLL, a PCR reaction was  
95 performed using FastStart DNA Taq-polymerase (Roche Diagnostics GmbH,  
96 Mannheim, Germany), cLL-specific forward primer 5'-  
97 GGATCCAAACCAACACAGATTTTTCC-3', and cLL-specific reverse primer 5'-  
98 GCGGCCGCAAACACTGGCAGACAACAAG-3' (Hogenkamp et al., 2006). The  
99 forward primer contained the BamHI restriction site sequence, and cLL reverse  
100 primer contained the NotI restriction site sequence. Amplification comprised of  
101 initial denaturation at 95°C for 2 min, followed by 40 cycles consisting of 95°C for  
102 30s, 49°C for 30s, 72°C for 1 min and a final extension at 72°C for 7 min. PCR-  
103 products were analyzed by agarose gel electrophoresis and purified with a QIAEX  
104 agarose gel extraction kit (Qiagen, Valencia, CA).

105

### 106 *2.3. Cloning and sequencing of the PCR products*

107 The purified PCR fragments were ligated into a pCR® 4-TOPO® plasmid vector  
108 (Invitrogen, Carlsbad, CA). Ligated plasmids were transfected into TOP10  
109 Escherichia coli cells by heatshock. Clones were selected by growth on Luria-Bertani  
110 broth (LB)-plates containing 100 µg/ml kanamycin. Positive clones were screened  
111 with PCR for correct product size and sequenced. Sequence reactions were  
112 performed using an ABI PRISM BigDye Terminator v3.0 Ready Reaction Cycle  
113 Sequencing kit (Applied Biosystems, Foster City, CA). All reactions were carried out  
114 in both directions using the T7 and T3 primer sites and separated on an ABI PRISM  
115 3100 fluorescent DNA sequencer (Applied Biosystems, Foster City, CA). cLL-  
116 inserts were isolated from positive clones using BamHI and NotI restriction sites and  
117 ligated into a pABC-cystatin-hisN vector with a cystatin signal peptide and an in-  
118 frame N-terminal His-tag.

119

#### 120 *2.4. Transfection*

121 HEK293-EBNA cells (ATCC CRL10852) were grown in 90% Freestyle (GIBCO;  
122 Invitrogen, Carlsbad, CA) and 10% Ca<sup>2+</sup>-free Dulbecco's modified Eagle's medium  
123 (DMEM) (GIBCO; Invitrogen, Carlsbad, CA), containing 5% FCS (Invitrogen), 1%  
124 pluronic (Sigma-Aldrich, St.Louis, CA), 10 mM HEPES, 4 mM L-glutamine, 200  
125 U/l penicillin G, 0.1 mg/l streptomycin and 50 µg/ml geneticin. Cells were  
126 maintained in exponential growth using Erlenmeyer flasks at 120 rpm on an orbital  
127 shaker mounted in a Reach-In CO<sub>2</sub> incubator (Clean Air Techniek, Woerden, The  
128 Netherlands). HEK293-EBNA cells were transfected using DNA-PEI (Polysciences,  
129 Warrington, PA) according to Durocher et al (2002). Briefly, 24 hrs before  
130 transfection, cells were seeded at 2.5 x 10<sup>5</sup>/ml in medium without FCS. The next day  
131 DNA-PEI complexes were formed by a 10 min incubation of plasmid DNA at 20



132  $\mu\text{g/ml}$  with PEI at  $40 \mu\text{g/ml}$  in Optimem (GIBCO; Invitrogen, Carlsbad, CA),  $25 \mu\text{l}$   
133 of this mixture was used for each ml of cell culture to be transfected. Small scale  
134 transfections ( $4 \text{ ml}$ ) were performed in 6 well plates, large scale transfections were  
135 performed in a 3L Erlenmeyer flask (Corning, NY).

136

### 137 *2.5. Purification of cLL*

138 The supernatant of transfected HEK293-EBNA cells was collected after 6 days and  
139 concentrated to a final volume of approximately  $250 \text{ ml}$  using a hollow fiber column  
140 (molecular-mass cut-off  $10 \text{ kDa}$ , Amersham Biosciences, Uppsala, Sweden).

141 Purification of cLL was performed by affinity chromatography (adapted from van  
142 Eijk et al., 2002). Briefly,  $1 \text{ ml}$  (bed-volume) mannan-sepharose (Sigma, St. Louis,  
143 MO), equilibrated in  $50 \text{ mM Tris-HCl}$ ,  $5 \text{ mM CaCl}_2$ , and  $0.05\%$  (vol/vol) Tween-80,  
144 pH  $7.4$  was added to the supernatant and  $\text{CaCl}_2$  was added to a final concentration of  
145  $5 \text{ mM}$ . The mixture was stirred overnight at  $4^\circ\text{C}$ . Sepharose beads were washed with  
146  $25 \text{ ml}$  washing buffer ( $50 \text{ mM Tris-HCl}$ ,  $5 \text{ mM CaCl}_2$ ,  $500 \text{ mM NaCl}$ ,  $0.05\%$  Tween-  
147  $80$ , pH  $7.4$ ). This washing procedure was repeated with  $25 \text{ ml}$  washing buffer  
148 without Tween-80. cLL was eluted with  $50 \text{ mM Tris-HCl}$ ,  $5 \text{ mM EDTA}$ , pH  $7.4$ . The  
149 eluted protein was concentrated using Amicon Ultra centrifugal filter units with a  
150 molecular weight cutoff of  $10 \text{ kDa}$  (Millipore, Billerica, MA), after which the buffer  
151 was changed to  $5 \text{ mM Tris-HCl}$ ,  $150 \text{ mM NaCl}$ , pH  $7.4$  by repetitive washing.

152 Purified cLL was stored in aliquots at  $-20^\circ\text{C}$ .

153

### 154 *2.6. Electrophoresis and Western Blot Analysis*

155 Proteins ( $0.1\text{-}1 \mu\text{g/lane}$ ) were analyzed by SDS-PAGE as described by Laemmli  
156 (1970) using  $10\%$  polyacrylamide gels. Protein bands were visualized by Coomassie

157 staining. For immunoblot analysis, proteins (0.1-1 µg/lane) were transferred  
158 electrophoretically from the gels onto nitrocellulose membrane. Immunostaining was  
159 performed using mouse anti-His<sub>6</sub> monoclonal antibody (Roche Diagnostics GmbH,  
160 Mannheim, Germany). Primary antibodies were detected by peroxidase-conjugated  
161 goat anti-rabbit immunoglobulin G (IgG) (Sigma, St. Louis, MO).

162

### 163 *2.7. Enzymatic deglycosylation of cLL*

164 N-deglycosylation treatment of cLL was performed using N-glycanase® PNGase F  
165 (ProZyme, Inc., San Leandro, CA) according to the manufacturer's instructions.  
166 Briefly, 45 µl cLL (230 µg/ml) was mixed with a 10 µl incubation buffer (0.25 M  
167 sodium phosphate, pH 7.0) 2.5 µl denaturation solution (2% SDS and 1 M β-  
168 mercaptoethanol). The sample was heated to 100°C for 5 min, after which the sample  
169 was cooled and 2.5 µl detergent solution (15% NP-40) was added. 2 µl of N-  
170 glycanase (5 U/ml) was added, and after 16 hr of incubation at 37°C, samples were  
171 concentrated using Microcon centrifugal filter units (MWCO 10 kDa) (Millipore,  
172 Billerica, MA). Samples were immediately processed for further analysis. O-  
173 deglycosylation treatment was performed using 1 µl 0.5 mU/µl O-Glycosidase  
174 (Roche Diagnostics GmbH, Mannheim, Germany) according to the protocol used for  
175 N-glycanase-PNG-ase. However, for O-glycosidase treatment the cLL storage buffer  
176 was changed to phosphate buffer (pH 7.0) using Microcon centrifugal filter units  
177 (MWCO 10 kDa) (Millipore, Billerica, MA). Deglycosylation by both N-glycanase-  
178 PNG-ase and O-glycosidase was carried out similarly to O-deglycosylation, but in  
179 this case both enzymes were added simultaneously.

180

### 181 *2.8. DIG-Glycan detection*

182 To assess possible glycosylation of cLL and enzymatically treated cLL, the DIG-  
183 Glycan detection kit (Roche Diagnostics GmbH, Mannheim, Germany) was used  
184 according to the manufacturer's instructions. Briefly, samples containing cLL were  
185 loaded onto gel and subsequently transferred from the gel onto nitrocellulose  
186 membrane.

187 The presence of glycoconjugates was assessed by labeling oxidized sugars with  
188 DIG-0-3-succinyl- $\epsilon$ -aminocaproic acid hydrazide, which was subsequently detected  
189 using anti-digoxigenin-AP and staining with NBT/X-phosphate.

190

### 191 2.9 *Bis (Sulfosuccinimidyl) suberate (BS)-crosslinking of cLL*

192 Crosslinking of cLL using Bis (Sulfosuccinimidyl) suberate (BS<sup>3</sup>) (Pierce, Rockford,  
193 IL) was performed according to the manufacturer's instructions. Briefly, the cLL  
194 storage buffer was changed to 10 mM HEPES buffer, pH 7.5 containing 3 mM  
195 EDTA using Microcon centrifugal filter units (MWCO 10 kDa) (Millipore, Billerica,  
196 MA). Final concentration of cLL was approximately 170  $\mu$ g/ml. BS<sup>3</sup> (50 mM) was  
197 added to the protein solution and incubated at room temperature for 30 min. The  
198 reaction was stopped by adding SDS-PAGE sample buffer to the mixture, which was  
199 subsequently heated to 100°C and loaded onto the gel for further analysis.

200

### 201 2.10. *MALDI TOF-TOF*

202 After protein separation on 12% SDS-PAGE and fixation in 50% methanol and 7%  
203 acetic acid, cLL was visualized using GelCode Blue Stain reagent (Pierce, Rockford,  
204 IL). The four visible bands were cut from the gel individually and subjected to in-gel  
205 tryptic digestion as previously described by van Balkom et al. (2005). Subsequently,  
206 these bands were identified by matrix-assisted laser desorbition/ionization (MALDI)

207 TOF-TOF analysis. The samples, dissolved in 0.1% acetic acid, were concentrated  
208 using  $\mu$ C18-ZipTips (Millipore) and eluted directly on the target plates in 1  $\mu$ l of a  
209 saturated solution of R-cyano-4-hydroxycinnamic acid in 50% (v/v) acetonitrile.

210 Data were acquired on a MALDI TOF-TOF instrument (Applied Biosystems 4700  
211 Proteomics Analyzer) in positive reflectron mode at a laser intensity of 3800 and a  
212 bin time of 0.5 ns.

213

### 214 *2.11. Carbohydrate specificity*

215 Polyacrylamide (PAA)-coupled glycoconjugates (~20% substitution; Lectinity,  
216 Lappeenranta, Finland) were coated (5  $\mu$ g/ml) in 0.2 M sodium cacodylate buffer  
217 (pH 9.2) on NUNC maxisorb plates (NUNC, Roskilde, Denmark) overnight at 4°C.  
218 Plates were blocked with 1% ELISA-grade BSA (Fraction V, fatty acid free;  
219 Calbiochem, San Diego, USA) in TSM (20 mM Tris-HCL pH 7.4, 150 mM NaCl, 2  
220 mM CaCl<sub>2</sub>, 2 mM MgCl<sub>2</sub>) and cLL was added (10  $\mu$ g/ml) for 2-3 hrs at 37°C in the  
221 presence or absence of 10 mM EDTA. After washing with TSM containing 0.1%  
222 Tween-20, 1  $\mu$ g/ml anti-His<sub>6</sub> antibody (Roche Diagnostics GmbH, Mannheim,  
223 Germany) was added for 1 hr at room temperature. Binding was detected using a  
224 peroxidase-labeled anti-mouse IgG antibody (Jackson, West grove, PA, USA) and  
225 measured in a Benchmark Microplate Reader using the Microplate Manager software  
226 (both from Biorad, Hercules, CA).

227

### 228 *2.12. Testing the activity of cLL against human isolates of IAV*

229

#### 230 *2.12.1. Virus preparations*

231 IAV was grown in the chorioallantoic fluid of 10-day-old chicken eggs and purified  
232 on a discontinuous sucrose gradient as described previously by Hartshorn et al.  
233 (1988). Virus stocks were dialyzed against PBS, aliquoted, and stored at -70°C.  
234 A/Phillipines/82 (H3N2) (Phil), and its bovine serum  $\beta$  inhibitor-resistant variant  
235 Phil/BS, were provided by Dr. E. M. Anders (Department of Microbiology,  
236 University of Melbourne, Melbourne, Australia). A/Puerto Rico/8/34 (H1N1) (PR-8),  
237 which lacks the high-mannose glycans on the haemagglutinin molecule, was  
238 provided by Dr. J. Abramson (Department of Pediatrics, Bowman Gray School of  
239 Medicine, Wake Forest University, Winston Salem, NC).

240

#### 241 *2.12.2. Neutralization of infectivity*

242 Madin-Darby canine kidney (MDCK) cell monolayers were prepared in 96-well  
243 plates and grown to confluence. These layers were then infected with a PBS<sup>++</sup> (PBS  
244 with 1 mM calcium and 0.5 mM magnesium; Gibco BRL, Grand Island, NY) -  
245 diluted IAV preparation (Phil strain) which was preincubated for 30 min at 37°C in  
246 the presence or absence (control) of increasing amounts of cLL. After exposure of  
247 the MDCK cells to the IAV or IAV/cLL mixture for 30 min at 37°C, the cells were  
248 washed three times in serum-free DMEM (Gibco BRL, Grand Island, NY)  
249 containing 1% (w/v) penicillin-streptomycin and subsequently incubated for 7 hrs at  
250 37°C. Next, the monolayers were washed, fixed, and FITC-labeled for IAV  
251 nucleoprotein as described previously by Hartshorn et al. (2002), after which  
252 fluorescent foci were counted.

253

#### 254 *2.12.3. Haemagglutination-inhibition assay*

255 Haemagglutination (HAA)-inhibition was measured by serially diluting cLL  
256 preparations in round-bottom 96-well plates (Serocluster U-Vinyl plates; Costar,  
257 Cambridge, MA) using PBS<sup>++</sup> as diluent (25 µl per well). After adding 25 µl of IAV  
258 solution, giving a final concentration of 40 HAA U/ml or 4 HAA U/well, the  
259 IAV/cLL mixture was preincubated for 15 min, followed by the addition of 50 µl of  
260 human erythrocyte suspension in PBS<sup>++</sup>. The entire procedure was performed at  
261 room temperature. The minimal concentration of cLL, required to fully inhibit the  
262 HAA caused by the virus, was determined by reading the plates after 2 hrs. HAA was  
263 detected as the formation of a pellet of erythrocytes.

264

### 265 *2.13. Statistical analysis*

266 Statistical analysis was performed using SPSS version 12.0.1 for Windows. Analysis  
267 of mean values between groups was carried out using Levene's Test for Equality of  
268 Variances. When equality of variance was assumed, mean values between groups  
269 were compared with an independent t-test, in which  $p < 0.05$  was considered to  
270 indicate statistical significance.

271 **3. Results**

272

273 *3.1. Production and characterization of recombinant cLL*

274 Preliminary results from the small scale transfections (not shown) showed that cLL  
275 was most effectively secreted using the mature peptide sequence ligated into the  
276 pABC-cystatin-hisN vector with a cystatin signal peptide and an in-frame N-terminal  
277 His-tag. Therefore, this vector was selected for large-scale (1 liter) transfections. The  
278 predicted amino-acid sequence of cLL expressed in this vector is shown in **Figure 1**.  
279 Affinity purification of the recombinant protein using mannan-sepharose yielded  
280 approximately 500-800 µg of protein per 1L batch (**Figure 2a**). Analysis of the  
281 purified product on SDS PAGE showed that four different bands varying in size  
282 between approximately 22 kDa and 27 kDa were present. Western Blot analysis  
283 using anti-His<sub>6</sub> antibody showed a positive signal for all four bands (**Figure 2b**) in  
284 the untreated cLL-sample (Lane 1) indicating that all four bands were recombinant  
285 products . To identify whether different glycosylation patterns of the protein could  
286 account for the observed multiplet, a DIG-glycan staining was performed. Results of  
287 this DIG-glycan detection are shown in **Figure 2c** and indicate that all of the 4  
288 recombinant protein bands contained sugar moieties. However, enzymatic treatment  
289 with N-glycanase / PNGase or O-glycanase of cLL did not result in a size shift (lane  
290 2-6), that would indicate loss of sugar moieties. The multimeric state of cLL was  
291 investigated by crosslinking experiments using Bis (Sulfosuccinimidyl) suberate.  
292 SDS PAGE analysis of the crosslinked cLL showed that monomeric cLL bands were  
293 not present anymore while two distinct high-molecular weight bands appeared upon  
294 BS<sup>3</sup> treatment of cLL (**Figure 3**).

295

296 3.2. *Analysis by MALDI-TOF-TOF*

297 MALDI-TOF-TOF analysis was performed in order to investigate the cause of  
298 appearance of four bands. However, the spectra of the trypsin-digested protein of all  
299 four bands were similar, and could not explain the size difference observed on SDS-  
300 PAGE. Several peaks in the spectra could be assigned to sequences in the  
301 recombinant protein, including the N- and C-terminus which were retrieved for all  
302 four bands (data not shown).

303

304 3.3. *Carbohydrate specificity*

305 Carbohydrate specificity of cLL was tested by use of PAA-coupled glycoconjugates.  
306 cLL was observed to preferentially bind to  $\alpha$ -mannose-PAA and trimannose-PAA  
307 (Man3), but not to any of the other glycoconjugates (**Figure 4**). Binding to  $\alpha$ -  
308 mannose-PAA and Man3-Paa was also tested in the presence of EDTA, which  
309 significantly reduced the binding, indicating that cLL binding to  $\alpha$ -mannose and  
310 Man3 is calcium-dependent.

311

312 3.4. *Testing the activity of cLL against human isolates of IAV*

313

314 3.4.1. *Neutralization of infectivity*

315 In order to investigate whether cLL was capable of protecting MDCK cell  
316 monolayers from infection by Phil-strain, Phil/BS-strain or PR-8 strain IAV, virus  
317 preparations were preincubated with increasing concentrations of cLL prior to adding  
318 them to the cells. No differences were observed in the number of fluorescent foci,  
319 indicating that cLL does not interfere with infection of these cells (data not shown).

320



321 *3.4.2. cLL mediated HAA-inhibition*

322 In the HAA-inhibition-assay, cLL was capable of inhibiting HA-activity of all three  
323 human isolates of IAV tested. The mean concentrations at which erythrocytes were  
324 no longer agglutinated by the virus are depicted in **Figure 5**. Phil/BS was inhibited at  
325 a much lower concentration of cLL than its parent strain Phil. Haemagglutination-  
326 activity of PR-8 was inhibited at a concentration comparable to that of Phil.

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327 **4. Discussion**

328

329 We previously reported the finding of cLL which, based on sequence homology, was  
330 predicted to be a C-type lectin (Hogenkamp et al., 2006). The sequence of its CRD is  
331 most similar to SP-A, but cLL does not contain a collagenous domain and therefore  
332 could not be classified as a collectin. To our knowledge, this protein lacks further  
333 similarity to other C-type lectins and should therefore be regarded as a novel type of  
334 lectin (Hogenkamp et al., 2006).

335 In this study, recombinant cLL was used to further characterize structural and  
336 functional properties of this lectin. It was found that cLL appeared as four differently  
337 sized products on SDS-PAGE gel (**Figures 2a and b**). No potential GPI-  
338 modification sites were found in the sequence, and since the sequence of cLL does  
339 not contain any potential N- glycosylation sites, it is highly unlikely that differential  
340 N-glycosylation could account for the appearance of four products instead of one.  
341 However, a positive signal was observed for all bands using the DIG-glycan  
342 detection kit (**Figure 2c**). Aspecific DIG-labeling or staining may explain this result.  
343 Accordingly, enzymatic treatment with N-Glycanase and/or O-glycosidase did not  
344 result in a size shift (**Figures 2b and c**), and no difference in intensity of DIG-glycan  
345 staining was observed between enzymatically treated samples. However, it cannot be  
346 excluded that cLL contains sugar moieties that are insufficiently removed by  
347 enzymatic treatment. As for O-linked glycans, it is known that these glycans are  
348 highly heterogeneous while in general O-glycosidases show a restricted specificity  
349 for subclasses of O-linked sugars. Therefore, we can not exclude the possibility that  
350 O-linked glycans are indeed present and result in heterogeneity of the cLL  
351 polypeptide chains as illustrated by results presented in **Figure 2**.

352 The four bands obtained by SDS-PAGE analysis on purified cLL were  
353 subjected to MALDI-TOF-TOF and resulted in identical spectra in which the C- and  
354 N-terminal fragments could be retrieved for all four bands, identifying all bands as  
355 (isoforms of) cLL.

356 This method is rather stringent, therefore it is possible that posttranslational  
357 modifications are removed during the procedure. Further analysis using 2D gel  
358 electrophoresis combined with liquid chromatography coupled to tandem mass  
359 spectrometry (LC-MS/MS) may reveal what causes the differences in mass. As this  
360 concerns a recombinant protein, it would be very interesting to find out whether the  
361 same size differences are present in native cLL.

362 In mammalian species, collectin monomers are known to organize into higher  
363 order multimers (Crouch et al., 1994; King et al., 1989). Crosslinking cLL led to the  
364 formation of two high molecular weight products (**Figure 3**), suggesting that this  
365 protein is capable of forming higher order subunits. The presence of heptad repeats  
366 in the cLL-amino acid sequence could allow for the formation of trimeric subunits  
367 (Hogekamp et al., 2006), characteristic for all C-type lectins. Furthermore, it is  
368 known that the carbohydrate-binding activity of C-type lectins is quite weak when  
369 they are in their monomeric form (Kishore et al., 1996). During the procedure of  
370 affinity-purifying cLL by use of mannan-sepharose beads, the beads were washed  
371 twice with a relatively large volume of washing buffer. These conditions would most  
372 likely be too stringent if carbohydrate-binding by cLL were weak, making it more  
373 likely that this protein is present as a trimer or a higher multimeric form.

374 Analysis of the carbohydrate-specificity of cLL revealed that the protein  
375 binds to  $\alpha$ -mannose and Man3 in a calcium-dependent manner, but other  
376 carbohydrates tested (including galactose) were not bound by cLL (**Figure 4**). It is

377 possible that the presence of a Glu-Pro-Ser motif in the CRD of cLL accounts for the  
378 relatively high specificity of cLL. Most other mannose type-collectins contain a Glu-  
379 Pro-Asn motif (Drickamer, 1992) and most SP-As contain a Glu-Pro-Arg motif,  
380 whilst retaining their preference for mannose over galactose (McCormack et al.,  
381 1994). However, Drickamer (1992) showed that substitution of one amino acid is  
382 enough to change the sugar binding specificity of C-type lectins. It would be  
383 interesting to determine if construction of a similar Glu-Pro-Ser motif in other C-type  
384 lectins would result in a comparable specificity for mannose.

385         The activity of cLL against human isolates of IAV was tested in viral  
386 inhibition assays. In HAA-inhibition assays cLL showed strong activity against the  
387 Phil/BS strain (**Figure 5**). Two other strains tested, Phil strain and PR-8, were less  
388 susceptible to cLL inhibition. The infection of MDCK cells by IAV could not be  
389 inhibited by cLL which may be explained by the, compared to collectins, relative  
390 weak interaction of cLL with these IAV strains. The change in equilibrium between  
391 cLL-bound and free virus particles could result in a constant supply of free IAV as  
392 MDCK cell infection draws free virus particles from the medium. This may explain  
393 the discrepancy between the results from the MDCK cell infections and the HAA-  
394 inhibition assays.

395         It is not yet clear what mechanism underlies the susceptibility of the different  
396 IAV strains with respect to the inhibition of haemagglutination by cLL. The Phil/BS  
397 strain (subtype H3N2) differs from the parent Phil strain, in that the high-mannose  
398 oligosaccharide overlying the sialic acid receptor-binding site of the HA molecule is  
399 absent (Hartley et al., 1992). It is possible that this reveals targets for cLL binding  
400 that are not available in the parent strain, resulting in increased binding. The PR-8  
401 strain also lacks high mannose glycans on the HA molecule (Schwarz and Klenk,

402 1981), but since this concerns a H1N1 subtype of IAV it is possible that differences  
403 in targets available on the HA molecule or the neuraminidase may result in decreased  
404 binding. It will be interesting to see what mediates cLL binding to IAV, since  
405 binding of SP-A (to which cLL is most similar) is thought to occur via binding of the  
406 sialic acid receptor of the virus to sialylated N-linked oligosaccharide present in the  
407 CRD of SP-A (Benne et al., 1995). It is possible that cLL, similar to SP-D (Hartshorn  
408 et al., 2000), binds to HA and neuraminidase in a C-type lectin-like manner, but the  
409 exact mechanism remains to be elucidated.

410

#### 411 **Conclusion**

412 The chicken lung lectin was successfully expressed in HEK293-EBNA cells. The  
413 purified protein proved to be a C-type lectin, as predicted from its sequence. Analysis  
414 of its carbohydrate specificity revealed that this protein has an unusual high  
415 preference for binding  $\alpha$ -mannose and trimannose. The results of the viral inhibition  
416 assays showed that cLL has moderate HAA-inhibition activity against the human  
417 IAV strains Phil and PR-8 and a strong inhibitory activity against Phil/BS. These  
418 results indicate that cLL could play an important part in the innate immune system of  
419 chickens.

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421

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507 **Figure Captions.**

508

509 **Figure 1. Predicted amino acid sequence of recombinant cLL.**

510 **1:** extra Gly-Ser-residues added after signal peptide; **2:** extra Gly-Ser-residues added as a result of  
 511 inserting the BamHI restriction site into the sequence; **3:** extra Alanine-residues added as a result of  
 512 inserting the NotI restriction site into the sequence

513

514 **Figure 2. Production and structural characterization of cLL.**

515 (A) Coomassie staining of cLL eluted from mannan-sepharose beads. (B) Western Blot-analysis of the  
 516 eluted cLL and enzymatically treated cLL using anti-His6. (C) DIG-glycan detection for both Sham-  
 517 treated and enzymatically treated cLL. (B&C): *1: untreated cLL, 2: Sham-treated N-Glycanase-*  
 518 *PNGase, 3: N-Glycanase-PNGase-treated cLL, 4: N-Glycanase-PNGase and O-glycosidase-treated*  
 519 *cLL, Lane 5: O-glycosidase-treated cLL, Lane 6: Sham-treatment O-glycosidase.*

520

521 **Figure 3. BS-crosslinking of cLL.**

522 *Left lane: untreated cLL; Right lane: crosslinked cLL.*

523

524 **Figure 4. Carbohydrate specificity of cLL.**

525 cLL binds to  $\alpha$ -mannose and Man3 in a calcium-dependent manner. *Man3: trimannose; LeX: Lewis X;*  
 526 *LeY: Lewis Y; LeA: Lewis A; H-type 2: Lewis H-type 2; H-type 3: Lewis H-type 3; LN: Gal- $\beta$ -*  
 527 *GlcNAc; LDN: GalNAc-beta-GlcNAc; chi-3: GlcNAc-GlcNAc-GlcNAc*

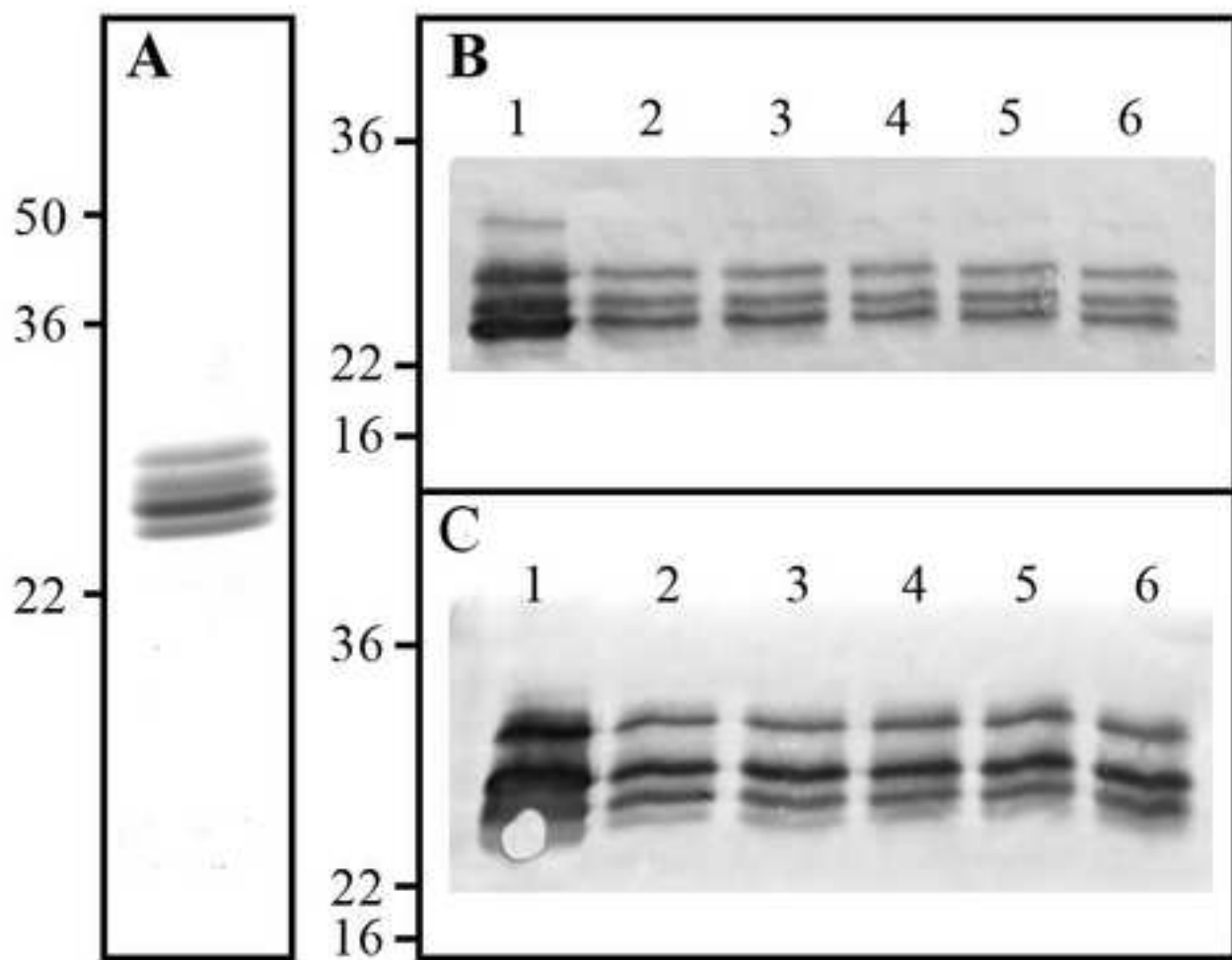
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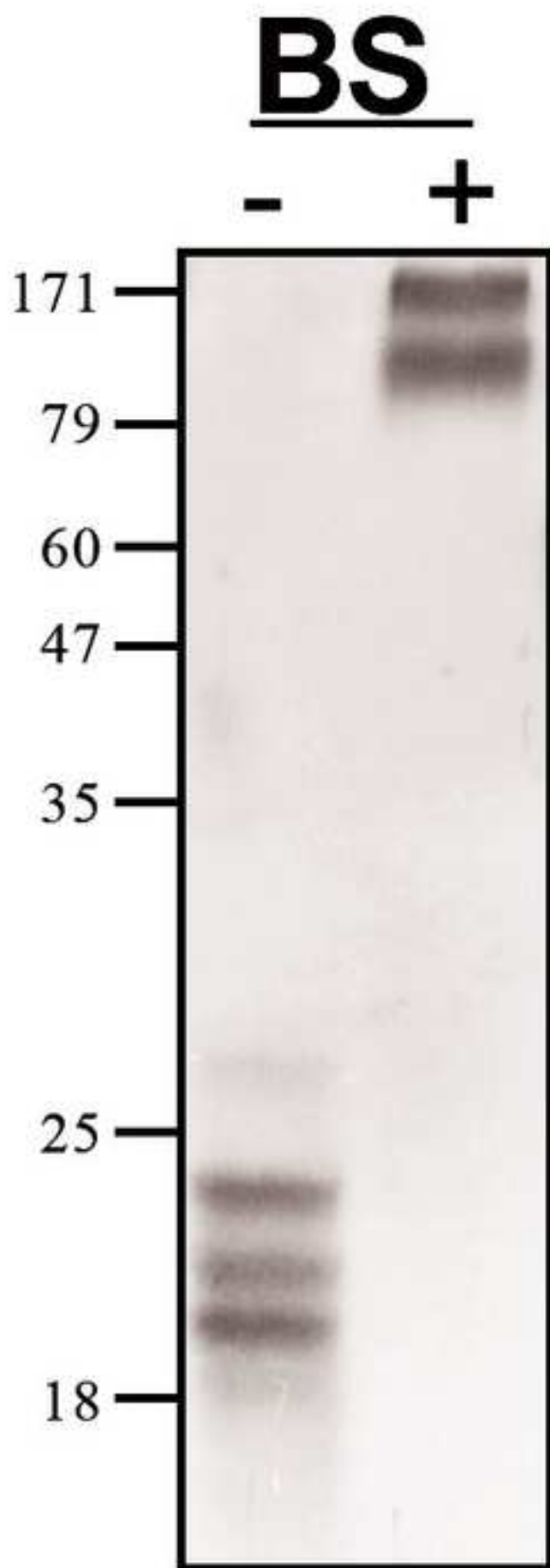
529 **Figure 5. Haemagglutination activity-inhibition by cLL**

530 Minimal concentration of cLL ( $\mu$ g/ml) necessary to inhibit HA-activity of human isolates of IAV.

531 Values are mean concentration  $\pm$  s.d. of three separate experiments.

<----- **Signal peptide** -----><-1-><- **HIS-tag** --><-2-><-----  
M-A-R-P-L-C-T-L-L-L-L-M-A-T-L-A-G-A-L-A-G-S-H-H-H-H-H-H-G-S-K-P-T-Q-I-F-P-  
----- **N-terminal region** -----> ----- **Neck Domain** -----  
V-P-G-F-K-A-E-R-G-I-S-Q-A-Y-L-P-G-F-P-S-V-A-G-S-E-M-D-D-A-V-L-Q-L-K-D-R-I-S-  
----->-----  
K-L-E-G-V-L-Y-L-Q-G-K-I-T-K-S-G-G-K-I-F-A-T-S-G-K-T-A-D-F-H-A-T-V-K-M-C-Q-E-  
----- **Carbohydrate Recognition Domain** -----  
A-G-G-C-I-A-S-P-R-N-A-D-E-N-A-A-I-L-H-F-V-K-Q-F-N-R-Y-A-Y-L-G-I-K-E-S-L-I-P-  
----->-----  
G-T-F-Q-F-L-N-G-G-E-L-S-Y-T-N-W-Y-S-H-E-P-S-G-K-G-E-E-E-C-V-E-M-Y-T-D-G-T-  
----->-3->  
W-N-D-R-R-C-N-Q-N-R-L-V-V-C-Q-F-A-A-A





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