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Research Article

A cold-active salmon goose-type lysozyme with high heat tolerance

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Abstract. The Atlantic salmon (*Salmo salar*) goose-type lysozyme gene was isolated and revealed alternative splicing within exon 2 affecting the signal peptide-encoding region. The lysozyme was produced in *Escherichia coli*, and the recombinant enzyme showed a high specific lytic activity that was stimulated by low or moderate concentrations of mono- or divalent cations. Relative lytic activities of 70 and 100 % were measured at 4 °C and 22 °C, respectively, and there was no detectable activity at 60 °C. However, 30 % activity was retained after heating the enzyme for 3 h at 90 °C. This unique combination of thermal properties was surprising since the salmon goose-type lysozyme contains no cysteines for protein structure stabilization through disulphide bond formation. The results point to a rapid reversal of inactivation, probably due to instant protein refolding.

Keywords. Atlantic salmon, immunity, lysozyme, goose-type, gene, protein, cold-active, thermal tolerance.

Introduction

Lysozymes (EC.3.2.1.17) encompass a group of enzymes that splits the β-1,4-glycoside bond between N-acetylmuramic acid (NAM) and N-acetylglucosamine (NAG) in peptidoglycans of bacterial cell walls. In animals three subclasses of lysozymes are found: the chicken (c-), goose (g-) (see [1, 2] for reviews) and invertebrate (i-) types [3]. While c-type lysozymes were described by Alexander Fleming already in the 1920 s [4], the presence of a different avian lysozyme was not discovered until 1967 [5], and the perception that these g-type lysozymes strictly belong in birds lasted until a few years ago, when g-type lysozymes or their genes were discovered in both fish and mammals [6–9]. To our surprise we then identified invertebrate g-type lysozyme genes and/or enzyme activity in two of three urochordate (or tunicate) classes [10], and very recently g-type lysozymes were discovered in bivalve molluscs [11]. The gene organization of chicken g-type lysozyme was published in 1991 [12], and later reports show that the gene structure is quite conserved and present in multiple copies in mammals and zebrafish as well as in some tunicates with unusual gene organisation [6, 9–11, 13]. In contrast to avian and mammalian g-type lysozymes, most fish counterparts investigated so far lack a signal peptide for secretion [6, 10, 13].

Although the similar hydrolysing activity of various lysozymes obviously points to analogous roles in innate immunity, the three types of animal lysozymes have no close sequence relatedness. A striking difference within the g-type subclass of lysozymes is the variation in cysteine content, ranging from zero to two in fish, four cysteines in the bird enzyme, four or seven

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in mammals and two to eleven cysteines in tunicates [5, 6, 9, 10]. Commonly, *g*-type lysozymes are recognised by the presence of a soluble lytic transglycosylase (SLT) domain, a domain defined by about 90 residues in the C-terminal part of transglycosylases in bacteria and shared by many prokaryotic and phage proteins [14, 15].

Functional characterizations of fish g-type lysozymes have been reported only to a minor extent. In this study we have cloned the Atlantic salmon g-type lysozyme gene and analyzed the recombinant protein for conditional enzymatic properties.

Materials and methods

Gene amplification. The database entry DW555874 represents an expressed sequence tag (EST) of salmon g-type lysozyme, and the derived oligonucleotides gSAL-F1 TGCAATAGATCAGTCAGAGGAA and -R1 GTACTGAATATTTTCACCATAGG were used to prime the amplification of the salmon lysozyme gene in which Atlantic salmon genomic DNA was used as the template in amplification over 33 cycles (94 °C for 15 s, 58 °C for 10 s and 72 °C for 4 min).

Amplification of the cDNA gene was based on a cDNA library of salmon ovaries, generously supplied by Tom Hansen (Norwegian College of Fishery Science, Tromsö, Norway). PCR primer sequences cSAL-F2 CAGGAAATCACCAAGAGAAAGAC and -R2 CACCATAGGTAGAATCAAAGACTG were designed, and amplification was achieved after 35 cycles of 94 °C for 30 s, 65 °C for 10 s and 72 °C for 30 s. The amplified cDNA gene was subsequently diluted and served as the template for a second PCR in which the primers rSAL-F3 CATATGGACATTACAAAGGTTGACACC and -R3 AAGCTTTCAATAACCCTGGCTTTTGAACC (introducing NdeI and HindIII restriction enzyme sites, respectively) directed amplification of the coding region of the gene minus the signal sequence during 18 cycles (94 °C for 15 s, 60 °C for 10 s and 72 °C for 30 s). A final step at 72 °C for 10 min was used for production of A-overhangs by the polymerase. All PCRs contained the following reaction mixtures in 100 µl final volumes: 200 µM dNTPs, 0.2 µM primers, 1× Advantage PCR buffer and 2 µl Advantage 2 polymerase (Clontech).

Cloning and recombinant expression. The amplification product carrying an A-overhang was subcloned using the TOPO TA Cloning kit (Invitrogen), and recombinant plasmids were digested with the NdeI and HindIII restriction enzymes (New England Biolabs). The released insert was ligated to expression vector pQE-2 (Qiagen) precut with the same restriction enzymes, and the ligation mixture was used to transform *E. coli* TOP10 cells (Invitrogen). The recombinant plasmid was finally transferred to the *E. coli* M15 (pREP4) expression host strain (Qiagen), and the resulting M15-pGM4 cells were cultivated in Luria Broth containing kanamycin (25 µg/ml) and ampicillin (100 µg/ml).

Three 1-litre cell cultures of M15-pGM4 were grown in the presence of 1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG; Sigma) at 23 °C to late log-phase in shaking flasks and then centrifuged. Precipitated cells were suspended in 40 ml lysis buffer (50 mM NaH_2PO_4, 300 mM NaCl, 10 mM imidazole) adjusted to pH 8, and two Complete Mini protease inhibitor cocktail tablets (Roche Applied Science) were added. The suspension was sonicated in 10 s pulses for 45 min on ice. After centrifugation the cleared lysate was subjected to affinity chromatography employing 6 ml Ni-NTA agarose (Qiagen) pre-equilibrated with lysis buffer. Washing with this buffer was followed by elution at increasing imidazole concentrations. Proteins were subsequently detected after SDS-PAGE electrophoresis in 4–12% NuPAGE Bis-Tris gel system (Novex) using an MES SDS running buffer. Proteins in gel were visualised by Coomassie G-250 staining, and migration lengths were compared to the Mark12™ protein standard (Novex).

Affinity-purified protein in solution was subjected to digestion by DAPase (Qiagen), an exoproteolytic dipeptidase for removal of His-tags in N-termini of proteins, and analysed by SDS-PAGE to confirm successful digestion. The vector-encoded sequence of Met-Lys-$(His)_6$ serves as a substrate for the DAPase, while the fused salmon lysozyme sequence represents an intrinsic stop for the peptidase. The processed recombinant protein solution was subjected once more to Ni-NTA chromatography to remove the His-tagged DAPase together with incompletely digested salmon lysozyme. Recombinant lysozyme free of His-tags was dialyzed against 50 mM ammonium acetate before lyophilisation, and the protein was dissolved in 50 mM Tris-HCl, pH 7.5. Details of the described method follow recommendations in the QIAexpressionist handbook (Qiagen).

Lysozyme activity measurements. Lysozyme activity was measured using lyophilised *Micrococcus lysodeikticus* cells (Sigma) as a substrate at a concentration of 0.2 mg/ml in 50 mM sodium acetate buffer (pH 5.2), 50 mM NaCl as previously described [16]. The activity was determined from the first 2 min of linear decrease in absorbance at 450 nm. One unit of lysozyme activity is defined as the amount of enzyme that catalyzes a reduction in absorbance of 0.001 min^{-1}. This method was used for all activity

measurements unless otherwise specified. For determination of pH for optimal activity, assay buffers of 10 and 50 mM sodium acetate and 5 and 20 mM sodium phosphate were used for pH ranges 3.6–5.6 and 6.0–8.0, respectively, after adjusting the buffer ionic strength using NaCl.

Miscellaneous. Sequence similarity and protein motif searches were performed using the FASTA [17] and InterProScan [18] programs, respectively, both online and hosted by the European Bioinformatics Institute (http://www.ebi.ac.uk/). Putative signal peptide in the encoded protein was predicted using the SignalP 3.0 server [19] at http://www.cbs.dtu.dk/services/SignalP. Oligonucleotide primer synthesis and DNA sequencing service were purchased from Eurogentec (Belgium) and Lark Technologies (UK).

Results and discussion

The Atlantic salmon g-type lysozyme (SalG) gene was amplified from an ovary cDNA library and from a genomic DNA isolate, and sequence alignment revealed the gene organization as shown in Fig. 1. The salmon gene is organized in five exons similar to g-type lysozymes in other vertebrates, and introns interrupt at identical intron phases within or between codons of homolog amino acids in these lysozymes. One exception from this is that exon 1 of SalG and other known fish g-type lysozyme genes carries a shorter protein-coding region (and a correspondingly longer exon 2) than in birds and mammals [6, 9, 12, 13]. The encoded salmon lysozyme has a predicted signal peptide of 21 amino acid residues. It is not clear to us why we previously failed to make this prediction in this particular protein sequence [10]. Protein motif scans showed that the salmon enzyme, like other g-type lysozymes, contains the soluble lytic transglycosylase domain defined by the bacterial Slt70 enzyme involved in balancing cell wall integrity [14].

Searches in EST databases for SalG sequences also identified entries such as EG918490 that, in contrast to entry DW555874, lacks the 5′ sequence of exon 2 encoding most of the signal peptide. Otherwise this EST is in full agreement with downstream sequences and the exon 1 sequence, thus not further changing the amino acid sequence of the encoded lysozyme. The missing sequence of EG918490 compared to the DW555874 sequence is underlined twice in Fig. 1, and what we originally believed was a truncated EST appears to be a product of alternative splicing within exon 2 in which a functional signal peptide sequence is removed. As seen in Fig. 1, this alternative splicing site still obeys general spliceosomal rules of intron 5′-GT donor and 3′-AG acceptor sites [20]. We found similar indications for alternative splicing in a few other fish species (*e.g.* zebrafish) whose genome sequences are known. Judged by the various EST entries in sequence databases, the SalG gene is expressed in head, kidneys, thymus, thyroid gland and skin, in addition to the ovaries (this study). The expression of a protein carrying or lacking the secretion signal may be a general need for both intracellular as well as extracellular lysozyme, or the respective SalG splicing forms may serve variable needs in specific tissues.

The SalG cDNA gene minus signal sequence was fused to a vector-contained sequence for an N-terminal His-tag and then expressed in *E. coli*, and Fig. 2 shows the image of an SDS-PAGE gel of the recombinant protein produced. Roughly estimated, 1 litre of culture produced 10–20 mg of recombinant SalG lysozyme when cells were grown and expression-induced at room temperature. The relative amount of recombinant protein produced dropped at higher temperatures. To reduce risks of artefact functionalities, the 8-residue His-tagged N-terminus was removed by successive steps of digestion with a diaminopeptidase. At least three steps of size reduction were observed after gel electrophoresis, and Fig. 2 also shows the gel image of the final cut protein.

The lytic activity of freshly produced recombinant salmon lysozyme on *M. lysodeikticus* cells was 9.4×10^5 units/mg protein at pH 5.2 and ionic strength 0.1. This extremely high specific activity is, however, significantly reduced during long-term storage at 4 °C, and we have not yet focused on optimizing storage conditions. The lytic activity of SalG lysozyme was further compared in buffers of varying pH, ionic strength and ion composition. The enzyme showed optimal activity at pH 5.2 and pH 6.8 at high and low ionic strengths, respectively (Fig. 3a), which is in the range previously reported for fish and avian g-type lysozymes [6, 21]. To our knowledge, the effect of cations on other g-type lysozymes has not previously been investigated. Addition of divalent cations at low concentrations increased the SalG activity by several fold, while higher concentrations of the monovalent cation sodium were required for similar stimulation (Fig. 3b). Divalent cations above 20 mM had a significant negative effect on enzyme activity.

Enzymatic activities of SalG and hen egg white lysozyme (HEWL) were studied at temperatures ranging from 4–80 °C at pH 5.2 and ionic strength 0.1 by adding enzyme (10 µl) to pre-heated *M. lysodeikticus* cell suspensions (990 µl). The results show that the SalG enzyme reached its peak at 22 °C and displayed a narrow temperature range of activity (Fig. 4a). The high lytic activity at 4 °C (70 % of maximum) demonstrates that this salmon enzyme is

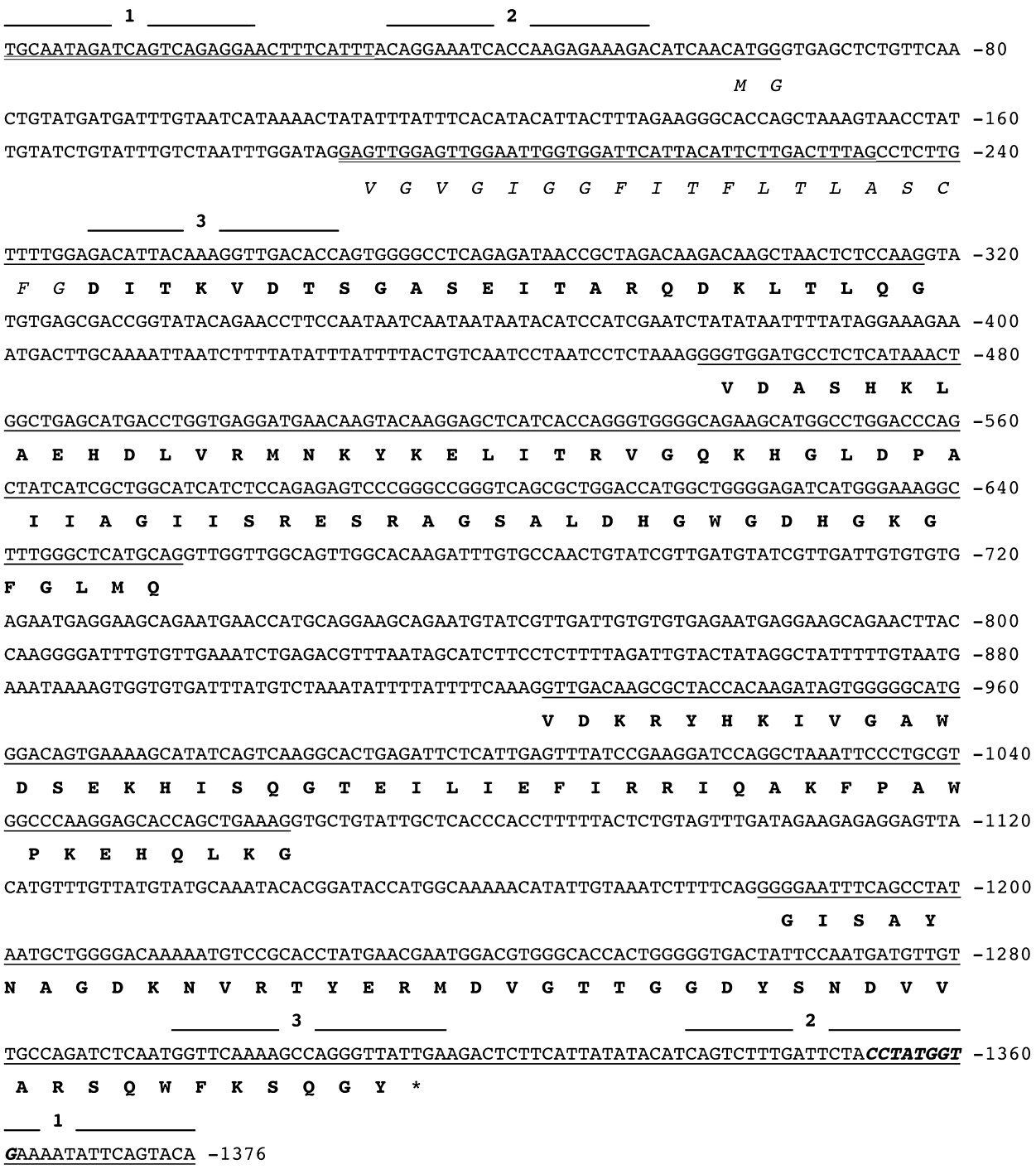

Figure 1. Genomic sequence of the salmon goose-type lysozyme gene (GenBank accession no. AM493682). The encoded protein sequence is shown below the corresponding DNA sequence. The deduced signal peptide is written in italics. Exons are underlined in accordance to sequence homology to EST sequence DW555874. The double-underlined sequence corresponds to the 5′ end of exon 2 (encoding most of the signal peptide), which is missing in EST sequence EG918490. The PCR primers used are indicated by numbered lines above the sense sequence: 1) genomic gene amplification, 2) primary cDNA amplification and 3) amplification of the encoding region minus signal peptide, representing the gene construct fused to the N-terminal His-tag in recombinant production.

cold-active. It has been shown elsewhere that marine [6] and avian [21, 22] g-type lysozymes have optimal temperatures in the range 25–30 °C and 30–40 °C, respectively.

Initial studies on effects of temperature on lysozyme activity after pre-incubating HEWL and SalG lysozyme for 15 min at temperatures ranging from 10 to 90 °C showed that neither enzyme was significantly

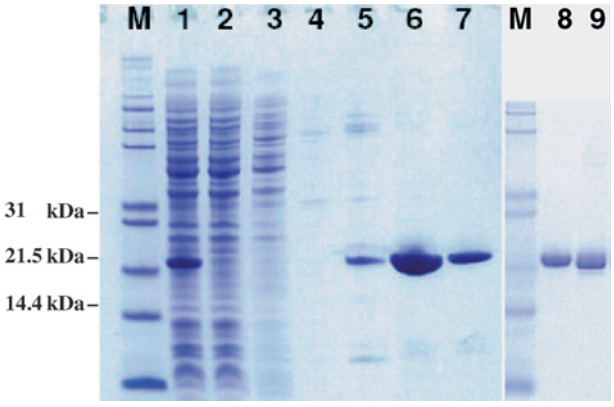

Figure 2. SDS-PAGE analysis of recombinant salmon g-type lysozyme expressed in *E. coli*, affinity-purified on Ni-NTA agarose and removal of His-tag. Lane contents are as follows: 1: Cleared lysate; 2: flow-through; 3–4: wash 10 and 20 mM imidazole; 5–7: eluates 40, 100 and 250 mM imidazole; 8: uncut lysozyme; 9: dipeptidase-cut lysozyme; and M: protein size markers.

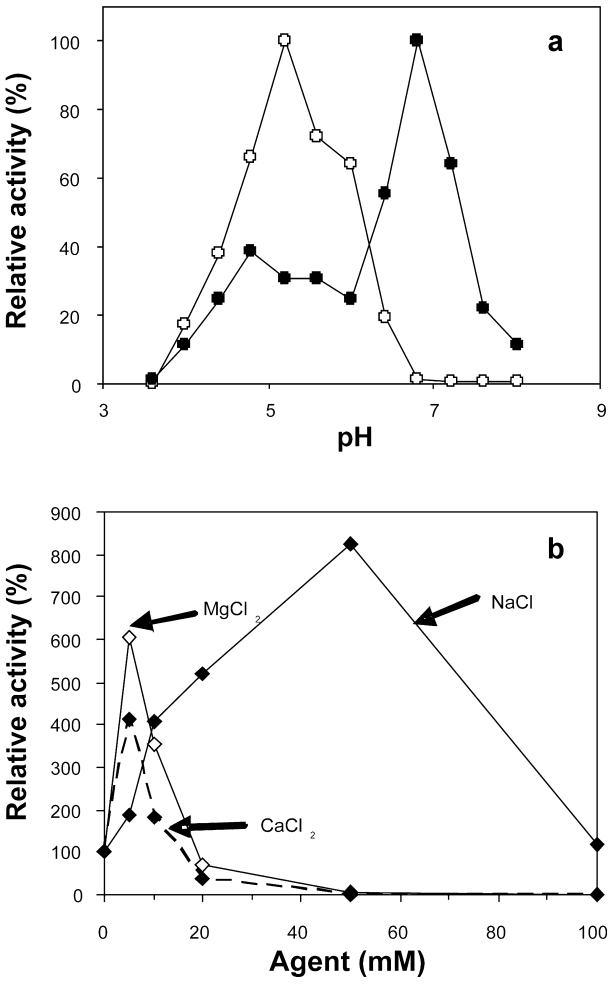

Figure 3. Effect of pH, ionic strength and ions on salmon g-type (SalG) lysozyme activity. (*a*) Activities of SalG lysozyme as measured at ionic strength 0.1 (○) and 0.03 (●) in sodium acetate buffer at pH 3.6 to 5.6 and sodium phosphate buffer at pH 6.0 to 8.0. Results are expressed as activity relative to the highest lysozyme activity measured at high and low ionic strength. (*b*) Effect of cations on the activity of SalG lysozyme. All values are normalized to the activity of enzyme (100%) at pH 5.2 and ionic strength 0.01.

affected by heat treatment (data not shown). Further investigations using prolonged incubations at 90 °C showed that remarkable 60 and 30% residual activities were monitored immediately after incubation of SalG lysozyme for 1 h and 3 h, respectively, and significantly lower survival of the HEWL activity was observed (Fig. 4b). The apparent temperature stability of the salmon enzyme is higher than reported for avian g-type lysozymes, although the latter contain two disulphide bridges. Bird lysozymes rapidly start loosing their activity upon heating; however, there are diverging results on thermal endurance, as the Cassovary enzyme lost all activity after 60 min at 80 °C [21], while higher temperature stability was reported for Rhea and Ostrich lysozymes [22]. Interestingly, heat inactivation of the His-tagged SalG lysozyme was much more efficient than with the native-like enzyme without the His-tag: the former became inactive after pre-heating for 15 min at 60 °C (not shown). We have no firm explanation yet as to why the N-terminal His-tag produces a thermointolerance for SalG. However, the salmon protein most likely possesses an extraordinary capacity to correctly refold its structure, and the His-tag may obstruct this refolding process.

The effect of temperature on SalG lysozyme and HEWL activity was also investigated by incubating the enzymes with substrate at a starting temperature of 60 °C and continuously monitoring the reaction as the temperature in the assay reaction solution fell (Fig. 4c). As expected, HEWL showed a high catalytic activity at the start of the reaction and a lower activity when the temperature decreased. Also consistent with the results presented above, SalG possessed no activity at the start but regained activity when the temperature decreased in the reaction mixture. It should be noted that the temperature did not go below 30 °C in this particular type of assay.

In sum, SalG displays high activity at low temperatures and loss of activity at elevated temperatures, it tolerates extensive pre-heating, and lysozyme activity is gradually regained during drop from non-permissive towards permissive temperature. Thus, this salmon lysozyme demonstrates cold activity but lacks heat lability. Cold-active enzymes are commonly characterized by increased catalytic efficiency at low temperatures, measured as k_{cat}/K_m ratio, and by significantly increased thermolability, which is believed to be a consequence of enhanced peptide chain flexibility (see [23–25] for reviews). In most (if not all)

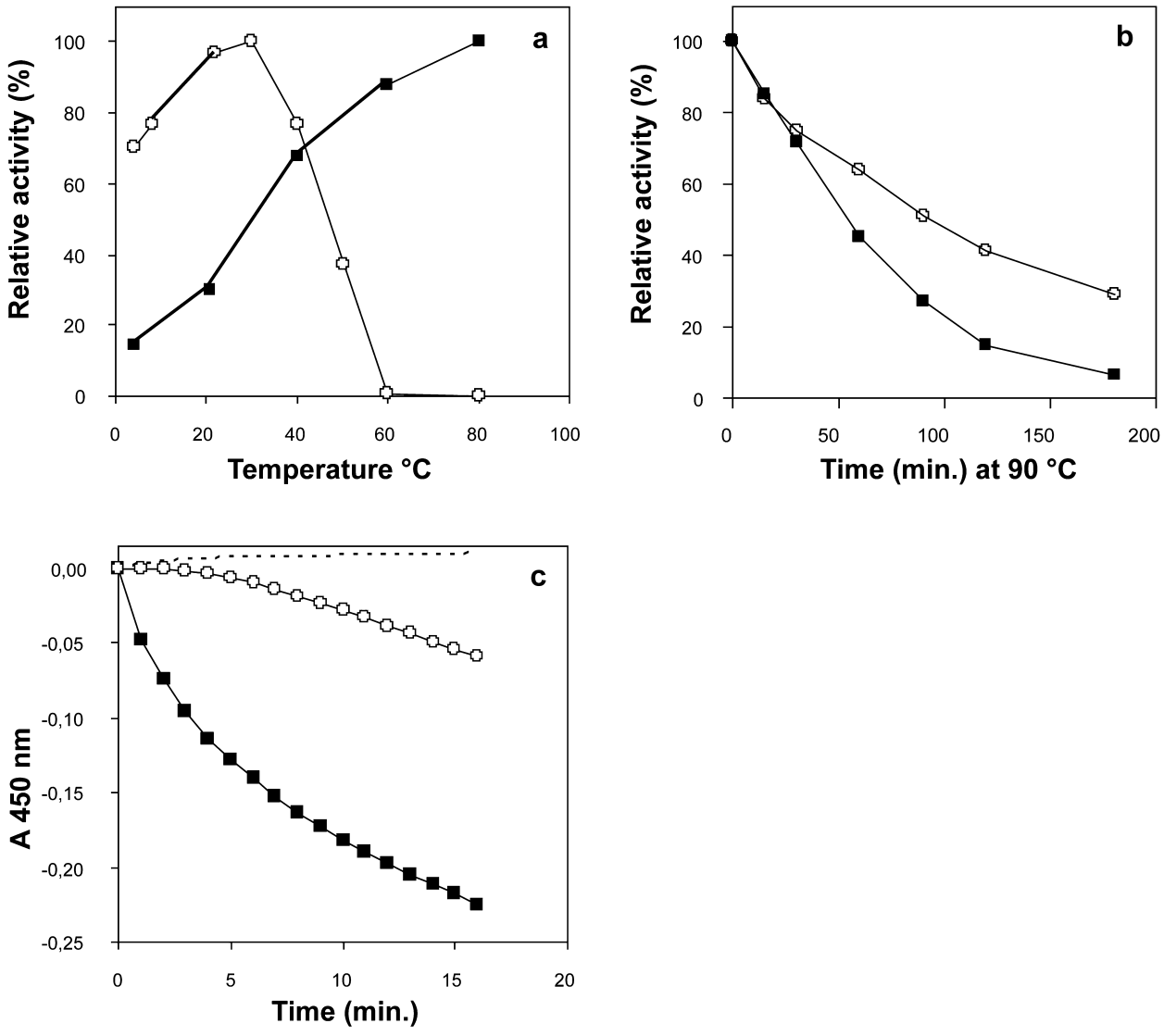

Figure 4. Effect of temperature on salmon g-type (SalG) lysozyme activity. The lytic activities of SalG lysozyme (○) and hen egg white lysozyme (HEWL, ■) were assayed at pH 5.2 and ionic strength 0.1. (*a*) Effect of assay temperature expressed as relative activity for each lysozyme. (*b*) Incubation at 90 °C for the indicated periods and immediate transfer to activity measurement at 22 °C. (*c*) Lytic activity in reactions with SalG lysozyme (5 μg) and HEWL (0.5 μg) was monitored starting at a temperature of 60 °C (time 0), which continuously decreased to 30 °C over a period of 16 min. The dotted line in (*c*) represents a negative control with no lysozyme present.

cases of cold-active enzymes, this thermolability has been shown to be irreversible [24, 25] in contrast to the demonstrated highly reversible effect on SalG. There are many reported cold-adapted enzymes from fish, mainly from Atlantic cod, and they are all easily inactivated at 50–70 °C, with no reported reversal of inactivation (exemplified in [26–28]). So far we are only aware of cold-active proteases from Atlantic salmon [29, 30]. Although cold adaptation of enzymes is generally associated with structure flexibility, these enzymes have a varying range of disulphide bridges [24, 25] that consequently do not represent a major determining factor for low temperature activity or thermolability.

High temperature endurance very similar to that of SalG lysozyme was reported a few years ago for a lytic C-terminal domain of a structural protein from a T7-like bacteriophage; the enzyme tolerated boiling and even autoclaving despite the lack of disulphide bonds [23]. We have only once previously, in a scallop i-type lysozyme carrying a high number of cysteines, seen the combination of low-temperature activity and high heat tolerance that we find in the salmon lysozyme [16]. The salmon enzyme is likely to go through a much more dramatic refolding process to reconstitute its activity after heating compared to the previously described scallop enzyme. In this study, testing of heating effects on the lysozymes routinely included

post-incubations on ice for several hours to allow potential refolding after the heating. We eventually discovered that this step was not necessary for SalG and that the enzyme regained most or all of its residual activity immediately after returning to the optimal temperature for activity.

In conclusion, Atlantic SalG lysozyme displays features of a cold-active as well as a thermo-tolerant enzyme. Our results indicate rapid refolding after heat-induced denaturation of the protein. Work is now being undertaken to study the protein 3D crystal structure and to monitor events of protein folding by differential scanning calorimetry.

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1 Jollès, J. and Jollès, P. (1984) What's new in lysozyme research? Mol. Cell. Biochem. 63, 165–189.
2 Jollès, P. (1996) Lysozymes: Model enzymes in biochemistry and biology. Birkhäuser, Basel.
3 Jollès, J. and Jollès, P. (1975) The lysozyme from *Asterias ruben.* Eur. J. Biochem. 54, 19–23.
4 Flemming, A. (1922) On a remarkable bacteriolytic element found in tissues and secretions. Proc. R. Soc. Lond. B 39, 306–317.
5 Canfield, R. E. and McMurry, S. (1967) Purification and characterisation of a lysozyme from goose egg white. Biochem. Biophys. Res. Commun. 26, 38–42.
6 Hikima J-i., Minagawa, S., Hirono, I. and Aoki, T. (2001) Molecular cloning, expression and evolution of the Japanese flounder goose-type lysozyme gene, and the lytic activity of its recombinant protein. Biochim. Biophys. Acta 1520, 35–44.
7 Savan, R., Aman, A. and Sakai, M. (2003) Molecular cloning of G type lysozyme cDNA in common carp (*Cyprinus carpio L.*). Fish Shellfish Immunol. 15, 263–268.
8 Yin, Z. X., He, J. G., Deng, W. X. and Chan, S. M. (2003) Molecular cloning, expression of orange-spotted grouper goose-type lysozyme cDNA, and lytic activity of its recombinant protein. Dis. Aquat. Org. 55, 117–123.
9 Irwin, D. M. and Gong, Z. (2003) Molecular evolution of vertebrate goose-type lysozyme genes. J. Mol. Evol. 56, 234–242.
10 Nilsen, I. W., Myrnes, B., Edvardsen, R. B. and Chourrout, D. (2003) Urochordates carry multiple genes for goose-type lysozyme and no genes for chicken- or invertebrate-type lysozymes. Cell. Mol. Life Sci. 60, 2210–2218.
11 Zou, H., Song, L., Xu, W. and Yang, G. (2005) Molecular cloning and characterization analysis of cDNA encoding g-type lysozyme from scallop (*Argopecten irradians*). High Tech. Lett. 15, 101–106.
12 Nakano, T. and Graf, T. (1991) Goose-type lysozyme gene of the chicken: Sequence, genomic organization and expression reveals major differences to chicken-type lysozyme gene. Biochem. Biophys. Acta 1090, 273–276.
13 Sun, B. J., Wang, G. L., Xie, H. X., Gao, Q. and Nie, P. (2006) Gene structure of goose-type lysozyme in the mandarin fish *Siniperca chuatsi* with analysis on the lytic activity of its recombinant in *Escherichia coli.* Aquaculture 252, 106–113.
14 Keck, W., Kazemier, B. and Engel, H. (1991) Murein-metabolizing enzymes from *Escherichia coli:* sequence analysis and overexpression of the *slt* gene, which encodes the soluble lytic transglycosylase. J. Bacteriol. 173, 6773–6782.
15 Koonin, E. V. and Rudd, K. E. (1994) A conserved domain in putative bacterial and bacteriophage transglycosylases. Trends Biochem. Sci. 19, 106–107.
16 Nilsen, I. W., Øverbø, K., Sandsdalen, E., Sandaker, E., Sletten, K. and Myrnes, B. (1999) Protein purification and gene isolation of chlamysin, a cold-active lysozyme-like enzyme with antibacterial activity. FEBS Lett. 464, 153–158.
17 Pearson, W. R. (1990) Rapid and sensitive sequence comparison with FASTP and FASTA. Meth. Enzymol. 183, 63–98.
18 Apweiler, R., Attwood, T. K., Bairoch, A., Bateman, A., Birney, E., Biswas, M., Bucher, P., Cerutti, L., Corpet, F., Croning, M. D. R., Durbin, R., Falquet, L., Fleischmann, W., Gouzy, J., Hermjakob, H., Hulo, N., Jonassen, I., Kahn, D., Kanapin, A., Karavidopoulou, Y., Lopez, R., Marx, B., Mulder, N. J., Oinn, T. M., Pagni, M., Servant, F., Sigrist, C. J. A. and Zdobnov, E. M. (2001) The InterPro database, an integrated documentation resource for protein families, domains and functional sites. Nucleic Acids Res. 29, 37–40.
19 Bendtsen, J. D., Nielsen, H., von Heijne, G. and Brunak, S. (2004) Improved prediction of signal peptides: SignalP 3.0. J. Mol. Biol. 340, 783–795.
20 Brunak, S., Engelbrecht, J., and Knudsen, S. (1991) Prediction of human mRNA donor and acceptor sites from the DNA sequence. J. Mol. Biol. 220, 49–65.
21 Thammasirirak, S., Torikata, T., Takami, K., Murata, K. and Araki, T. (2001) Purification and characterization of goose type lysozyme from Cassovary (*Casuarius casuarius*) egg white. Biosci. Biotechnol. Biochem. 65, 584–592.
22 Pooart, J., Torikata, T. and Araki, T. (2005) Enzymatic properties of Rhea lysozyme. Biosci. Biotechnol. Biochem. 69, 103–112.
23 Smalås, A. O., Schrøder Leiros, H. K., Os, V. and Willassen, N. P. (2000) Cold-adapted enzymes. Biotechnol. Annu. Rev. 6, 1–57.
24 Feller, G. (2003) Molecular adaptations to cold in psycrophilic enzymes. Cell. Mol. Life Sci. 60, 648–662.
25 Siddiqui, K. S. and Cavicchioli, R. (2006) Cold-adapted enzymes. Annu. Rev. Biochem. 75, 403–433.
26 Strætkvern, K. O., Raae, A. J. and Walther,, B. T. (1992) Characterization of a pancreatic DNase from pyloric caeca of Atlantic cod (*Gadus morhua*). Fish Physiol. Biochem. 9, 439–452.
27 Ásgeirsson, B., Hartemink, R. and Chlebowski, J. F. (1995) Alkaline phosphatase from Atlantic cod (*Gadus morhua*). Kinetic and structural properties which indicate adaptation to low temperatures. Comp. Biochem. Physiol. Biochem. 110, 315–329.
28 Lanes, O., Leiros, I., Smalås, A.O. and Willassen, N. P. (2002) Identification, cloning, and expression of uracil-DNA glycosylase from Atlantic cod (*Gadus morhua*): characterization and homology modeling of the cold-active catalytic domain. Extremophiles 6, 73–86.
29 Outzen, H., Berglund, G. I., Smalås, A. O. and Willassen, N. P. (1996) Temperature and pH sensitivity of trypsins from Atlantic salmon (*Salmo salar*) in comparison with bovine and porcine trypsin. Comp. Biochem. Physiol. Biochem. 115, 33–45.
30 Berglund, G. I., Smalås, A. O., Outzen, H. and Willassen, N. P. (1998) Purification and characterization of pancreatic elastase from North Atlantic salmon (*Salmo salar*). Mol. Mar. Biol. Biotechnol. 7: 105–114.
31 Lavigne, R., Briers, Y., Hertveldt, K., Robben, J. and Volckaert, G. (2004) Identification and characterization of a highly thermostable bacteriophage lysozyme. Cell. Mol. Life Sci. 61, 2753–2759.