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Molecular cloning of the first metazoan β-1,3 glucanase from eggs of the sea urchin *Strongylocentrotus purpuratus*

(cortical granule)

ERIC SCOTT BACHMAN* AND DAVID R. MCCLAY†

Developmental Cell and Molecular Biology Group, Box 91000, Duke University, Durham, NC 27708

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ABSTRACT **We report the molecular cloning of the first β-1,3 glucanase from animal tissue. Three peptide sequences were obtained from β-1,3 glucanase that had been purified from eggs of the sea urchin *Strongylocentrotus purpuratus* and the gene was cloned by PCR using oligonucleotides deduced from the peptide sequences. The full-length cDNA shows a predicted enzyme structure of 499 aa with a hydrophobic signal sequence. A 3.2-kb message is present in eggs, during early embryogenesis, and in adult gut tissue. A polyclonal antibody to the native 68-kDa enzyme recognizes a single band during early embryogenesis that reappears in the adult gut, and recognizes a 57-kDa fusion protein made from a full-length cDNA clone for β-1,3 glucanase. The identity of this molecule as β-1,3 glucanase is confirmed by sequence homology, by the presence of all three peptide sequences in the deduced amino acid sequence, and by the recognition of the bacterial fusion protein by the antibody directed against the native enzyme. Data base searches show significant homology at the amino acid level to β-1,3 glucanases from two species of bacteria and a clotting factor from the horseshoe crab. The homology with the bacteria is centered in a 304-aa region in which there are seven scattered regions of high homology between the four divergent species. These four species were also found to have two homologous regions in common with more distantly related plant, fungal, and bacterial proteins. A global phylogeny based on these regions strongly suggests that the glucanases are a very ancient family of genes. In particular, there is an especially deep split within genes taken from the bacterial genus *Bacillus*.**

The class of enzymes known as β-1,3 glucanases are well-characterized in fungi, bacteria, and plants. In these kingdoms, β-1,3 glucanases function in various extracellular capacities including autocatalysis of extracellular matrix glucans (fungi), pathogenic digestion of cell walls (bacteria), and as inducible defense enzymes in plants (1–5). These glycosyl hydrolases are specific for O-linked glycosyl bonds found in a variety of substrates, including β-glucans found in fungal cell walls (6, 7). Many of the enzymes in plants and bacteria have been cloned and several have been characterized by crystallization, but until now no metazoan β-1,3 glucanase sequences have been described.

In the animal kingdom, the known distribution of β-1,3 glucanases is restricted to the eggs and digestive tract of echinoderms (8). The enzymes in these two cell types are of the same molecular weight and are antigenically similar (9). In the gut, the enzyme is presumed to catalyze the degradation of ingested algal β-glucans. Why β-1,3 glucanase is in sea urchin eggs and is released at fertilization remains unclear. The enzyme is exocytosed from cortical granules 30 sec after fertilization, and active enzyme can be recovered from both young embryos and the perivitelline space surrounding the embryo (10). All but one of 13 species of sea urchin studied have β-1,3 glucanase stored in cortical granules in unfertilized eggs (11). Nevertheless, its endogenous substrate and function are unknown.

To gain some insight of possible function in the egg, we wished to establish the molecular structure of sea urchin β-1,3 glucanase. We purified oocyte β-1,3 glucanase enzyme to homogeneity, acquired peptide sequence from three proteolytic fragments, cloned a family of overlapping cDNAs for sea urchin β-1,3 glucanase, and sequenced the entire cDNA for this gene. The predicted protein sequence shows remarkable similarity to bacterial enzymes, particularly from *Bacillus circulans* and the marine eubacterium *Rhodothermus marinus* (12, 13). Based on this molecular homology and previous biochemical studies, we believe this enzyme may function in a capacity similar to its prokaryotic relatives. The relationship between sea urchin and bacterial β-1,3 glucanases is curious especially since there are no other animal glucanases described. The possibility was considered that this enzyme arose in echinoderms by horizontal transfer of genetic information from commensal gut bacteria. However, using phylogenetic arguments, the actual evolutionary origin of this protein appears to be quite ancient and the notion of horizontal transfer is not well-supported by these arguments.

MATERIALS AND METHODS

Animals. Sea urchins (*Strongylocentrotus purpuratus*) were purchased from Marinus (Long Beach, CA). Eggs were obtained by injecting sea urchins with 0.5 M KCl to induce spawning and collected by settling in artificial seawater. A total of ≈500 ml of packed eggs were obtained for protein purification.

Enzyme Purification. β-1,3 glucanase was purified as described (14). Briefly, embryos and gametes were washed 3× in artificial seawater and homogenized in a 50 mM $NaPO_4$/200 mM NaCl, pH 7.0, buffer containing a protease inhibitor cocktail of 0.1% phenylmethylsulfonyl fluoride, leupeptin, and aprotinin. Egg homogenates were centrifuged and the supernatant was run over a gluconolactone affinity column, washed, eluted with 2M NaCl/50% PEG 6000, and dialyzed against K_2HPO_4, pH 7.2. Typical enzyme purification was 800- to 1000-fold from whole homogenates of oocytes and adult guts. One unit of activity was defined as the amount of enzyme that produces 10 nm of glucose per minute at 37°C. Enzyme purity was determined by SDS/PAGE, followed by silver or Coomassie blue staining (15). Polyclonal antibody against the native 68-kDa protein was available in our lab (16).

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Data deposition: The sequence reported in this paper has been deposited in the GenBank data base (accession no. U49711).

*Present address: Department of Medicine, Beth Israel Hospital, 330 Brookline Avenue, Boston, MA 02215.
†To whom reprint requests should be addressed. e-mail: dmcclay@acpub.duke.edu.

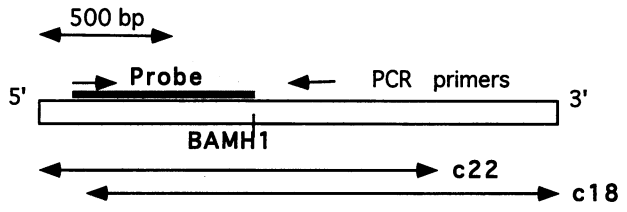

FIG. 1. Diagram of cloning strategy for sea urchin β-1,3 glucanase. Primers used to amplify a 1-kb fragment are indicated, as is the 800-bp probe used in library screening and Northern blotting that resulted from *Eco*RI/*Bam*HI digestion of the PCR product.

Peptide Sequencing. Purified enzyme as described above was gel purified on 7.5% SDS/PAGE, briefly stained in aqueous Coomassie blue, and destained in water. The single bands at 68 kDa were cut out, equilibrated in 10 mM Tris buffer pH 6.8, and reloaded into lanes of a 15% polyacrylamide gel with 1 mM EDTA. To each well containing ≈50 mg of enzyme, 0.02 ml buffer A (0.1% SDS/1 mM EDTA/2.5 mM DTT/0.125 M Tris, pH 6.8) was added. These slices were overlaid with 20% glycerol in buffer A, and finally with 10% glycerol containing buffer A and 1 mg of V8 protease (Boehringer Mannheim). The gel was run through the stacker and stopped for 1 hr to allow digestion to proceed. This completed, the gel was run, then blotted onto polyvinylidene difluoride-immobilon membrane (Millipore) and stained with Coomassie blue. Peptide sequencing was performed on an Edman Automated Sequencing Apparatus.

Oligonucleotide Synthesis. Oligonucleotide primers for the PCR were prepared on an automated synthesizer. Primers were designed to the intact N-terminal amino acid sequence YDVKNPEISL and the 17-kDa peptide NADIKDADG. These were, respectively, 5′ (with *Bam*HI site) CGGGATCCCGTA(C/T)GA(C/T)GT(A/C/G/T)AA(A/G)AA(C/T)CC(A/C/T)GA(A/G)AT(A/C/T)(A/T)C/G and 3′ (with an *Eco*RI site) CGGAATTCCGCC(A/G)TCIGC(A/G)TC(C/T)TT(A/T/G)AT(A/G)TCIGC(A/G)TT. I indicates inosine at that residue. RNA isolation was performed using a kit for generation of poly(A)$^+$ RNA from wet preparations of tissue (Pharmacia).

PCR. Poly(A)$^+$ RNA from eggs, ovaries, and immature oocytes of *S. purpuratus* was reverse transcribed to cDNA using standard reagents (New England Biolabs). As an internal control for the authenticity of the starting cDNA, we amplified a 450-bp fragment using published primers specific for the sea urchin sperm receptor (17). PCR reactions were carried out for 40 cycles with 1 min at 94°C/1 min at 50°C/1 min at 72°C using 10 μl cDNA, 5 μM of each primer, and PCR buffer solution containing 1.5 mM Mg. The 980-bp product was diluted 100×, reamplified as above, and digested by *Eco*RI and *Bam*HI and cloned into the Bluescript plasmid (Stratagene).

Sequencing. The dideoxy method was used (United States Biochemical). A transposon-assisted method was used to generate subclones for sequencing (18). The 800-bp product

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                                                ATCGTTTGATGTACGAGGATCAGTTAGTCGTTAGGACACATTCGTCAAG      50
ATGGTCGACTTAAGACACGTATTGGCTTTGCTACCTGTTATATTAGTTTCGATTAATGCGTATGATGTTAAGAATCCTGAGATC                  134
 M  V  D  L  R  H  V  L  A  L  L  P  V  I  L  V  S  I  N  A  Y  D  V  K  N  P  E  I                   28
TCACTCTTGACTCCTCGGGGAATCCGATTTGCATACCCAGATGAATCTGGCACAACCCTGGTAGCTTTTCAYTATAACATCAAT                  218
 S  L  L  T  P  R  G  I  R  F  A  Y  P  D  E  S  G  T  T  L  V  A  F  H  Y  N  I  N                   56
ACACCACTATCTGGTGTTGGGGCTGGTCAATATAACTATGATGTCACTACTACAACAGATGAATACTTCGTCCATGAAAATAGA                  302
 T  P  L  S  G  V  G  A  G  Q  Y  N  Y  D  V  T  T  T  T  D  E  Y  F  V  H  E  N  R                   84
GACGTGGATGTTGAGAACGGTGATGTGGTGTACTACTGGGTGTATACAGTTTACACGGGCCTTGGTTACCAACTAACTGACCAA                  386
 D  V  D  V  E  N  G  D  V  V  Y  Y  W  V  Y  T  V  Y  T  G  L  G  Y  Q  L  T  D  Q                  112
TCATGGACAGCATCTGAAACTACAGAAGCTCCTGCGACCAATCCCCCTGCAACCGAATCCCCTGTGACCAATGCCCCTGCAACC                  470
 S  W  T  A  S  E  T  T  E  A  P  A  T  N  P  P  A  T  E  S  P  V  T  N  A  P  A  T                  140
GAATCCCCTAACCCAGGCACCGGCACGACACAGTCTAGTGGTGGAGGTACGTCGCAGTGTAGCATGTATCCTTGCGATGCAGCA                  554
 E  S  P  N  P  G  T  G  T  T  Q  S  S  G  G  G  T  S  Q  C  S  M  Y  P  C  D  A  A                  168
TGTGACATGTCCACTCCACCTTGCAATGGTCTCATCTTCCAAGAAGAGTTTGATTCTTTCAATCTTGACATCTGGGAGCATGAA                  638
 C  D  M  S  T  P  P  C  N  G  L  I  F  Q  E  E  F  D  S  F  N  L  D  I  W  E  H  E                  196
ATGACTGCCGGAGGAGGAGGGAACTGGGAATTTGAATATTATACCAACAACCGATCAAACAGCTATGTTCGGGATGGAAAACTC                  722
 M  T  A  G  G  G  G  N  W  E  F  E  Y  Y  T  N  N  R  S  N  S  Y  V  R  D  G  K  L                  224
TTCATAAAACCAACACTTACCACCGATAAATTGGGAGAGGGTTCGCTGTCATCTGGAACGTTAGACCTTTGGGGTTCATCCCCA                  806
 F  I  K  P  T  L  T  T  D  K  L  G  E  G  S  L  S  S  G  T  L  D  L  W  G  S  S  P                  252
GCTAACTTGTGTACCGGTAATGCATGGTATGGATGTTCGCGAACGGGATCCAATGATAATCTATTGAATCCGATACAGTCTGCT                  890
 A  N  L  C  T  G  N  A  W  Y  G  C  S  R  T  G  S  N  D  N  L  L  N  P  I  Q  S  A                  280
CGTCTACGTACTGTCGAATCTTTCTCATTCAAGTATGGACGACTGGAGGTTGAGGCTAAGCTACCCACTGGTGATTGGCTTTGG                  974
 R  L  R  T  V  E  S  F  S  F  K  Y  G  R  L  E  V  E  A  K  L  P  T  G  D  W  L  W                  308
CCAGCTATCTGGCTTTTGCCKAAGCATAACGGATATGGAGARTGGCCTGCCTCYGGAGAAATAGATCTGGTTGAAAGCAGAGGT                 1058
 P  A  I  W  L  L  P  K  H  N  G  Y  G  E  W  P  A  S  G  E  I  D  L  V  E  S  R  G                  336
AACGCTGATATCAAAGAYGCGGATGGTTTRTCAGCTGGRGTAGATCAGATGGGCTCTACTATGCATTGGGGACCATTCTGGCCT                 1142
 N  A  D  I  K  D  A  D  G  L  S  A  G  V  D  Q  M  G  S  T  M  H  W  G  P  F  W  P                  364
CTGAACGGATATCCAAAGACACACGCAACAAAGTTCTACGTGGATGATGAGCTTCTATTGAATGTTGATCCTGCTACAGGCTTT                 1226
 L  N  G  Y  P  K  T  H  A  T  K  F  Y  V  D  D  E  L  L  L  N  V  D  P  A  T  G  F                  392
TGGGACTTGGGTGAATTTGAGAATGATGCACCAGGCATTGACAATCCATGGGCTTACAATCCTAATAAACTCACTCCATTCGAT                 1310
 W  D  L  G  E  F  E  N  D  A  P  G  I  D  N  P  W  A  Y  N  P  N  K  L  T  P  F  D                  420
CAAGAGTTCTACTTGATCCTGAACGTAGCTGTAGGAGGAGTCAACTATTTCGGTGAYGGGYTGACATACACCCCAGCTAAACCA                 1394
 Q  E  F  Y  L  I  L  N  V  A  V  G  G  V  N  Y  F  G  D  G  L  T  Y  T  P  A  K  P                  448
TGGTCTAATGACTCCCCTACGGCCTCTAAGGATTTCTGGTCTGACTTTAACACTTGGTATCCTACATGGAATGGTGAGGAAGCA                 1478
 W  S  N  D  S  P  T  A  S  K  D  F  W  S  D  F  N  T  W  Y  P  T  W  N  G  E  E  A                  476
GCAATGCAGGTCAACTATGTYCGTGTRTACGCAGAACCCGGGCAGACAACATATCACCTACGTGATCGTTAAATAATGTACCAT                 1562
 A  M  Q  V  N  Y  Y  V  R  V  Y  A  E  P  G  Q  T  T  Y  H  L  R  D  R                            499

CAAACCTGCCGTGATCAGTAAAAGAATCACGCAAATTAGCCCCATAATTTCCATATTAAAAGAAGGTAATTTCTAATGTTGGGA
TTCCGATGAAATAGAAAACCATTTTGTAGATGTTCGTAAATATAGCATGATCTTTTAATATTATAGTCTATATAATATCCAATT
ATCCAAAAGGACGCGTACAACATGAGTAGTTTACATGACTACATATGACAATCGAAACTATCAGAGAAATCTAC                         1805
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FIG. 2. Nucleotide sequence and predicted amino acid sequence of β-1,3 glucanase from *S. purpuratus*. The start Met is preceeded by a consensus Kozak sequence (boxed) at nucleotide 50, with a 20 aa hydrophobic signal sequence, followed by the predicted open reading frame. Peptide sequences derived from the native protein earlier are underlined (MACVECTOR). The predicted 499 aa sequence has a stop codon that is followed by multiple stop codons in all reading frames.

FIG. 3. Closest relatives of *S. purpuratus* glucanase. Underlined regions were also homologous to a wider taxonomic range of genes (Fig. 4). The genes from the cyanobacteria *Bacillus* (sp|P23903) and *Rhodothermus* (pir|S48201) are glucanases, whereas the gene from the horseshoe crab (*Tachypleus)* is a blood clotting factor (pir|A49878). These genes were identified by a blastp search (23). Alignments were performed using CLUSTAL V (20) using Identity matrix and a gap weight of 10. A second glucanase was also been found in *B. circulans*, but is so closely related to the *Bacillus* sequence shown here that it was not included.

from the digestion with *Eco*RI/*Bam*RI of the 1-kb original PCR product was used to screen a Lambda Zap library made to ovary/oocytes of *S. purpuratus*. DNA was prepared into probe using a [^{32}P]dCTP and kit reagents (Stratagene). Clones were derived from two rounds of screening, which produced cDNAs that covered the open reading frame plus portions of both 5′ and 3′ untranslated regions of the cDNA.

Northern Blot Analysis. Poly(A)$^+$ RNA (≈5 μg per lane) was isolated from eggs and embryos of *S. purpuratus* at 16, 19.5, 24.5, 31.5, and 37.5 hr of development, and from *Lytechinus variegatus* eggs, ovary, and adult gut tissue. Samples were electrophoresed on a 1.2% agarose/formaldehyde gel, blotted onto a nylon membrane, and hybridized using the 800-bp PCR product as a probe (19). The blot was washed 3× at 50°C for 15 min in 0.2× SSC/0.1% SDS and exposed overnight. As a control, the blot was stripped and reprobed with a ubiquitin fragment from *Lytechinus pictus*.

Generation of Fusion Proteins. Clone 18 from screening above was ligated into the *Eco*RI site of pGEX-1, transformed, and induced to form fusion proteins. This protein was extremely insoluble, and was resolubilized in 8 M urea before SDS/PAGE and Western blot analyses using the antibody made to the 68-kDa intact, native protein.

Molecular Evolution Analysis. The amino acid sequences were aligned using the CLUSTAL V program (20). Alignments based on three different weighting schemes were compared, and the 304 aa stretch was consistently aligned across all three weighting schemes [equal weighting, Dayhoff PAM 100, PAM 250 (20)].

RESULTS

Cloning of β-1,3 Glucanase. Degenerate oligonucleotides were synthesized and used in the PCR reaction that produced a 980-bp product. The 800-bp product after *Eco*RI/*Bam*RI digestion was cloned into Bluescript. The deduced amino acid sequence, based on the DNA sequence, included a sequence identical to the peptide sequence obtained earlier from the native protein. This clone was then used to screen an ovary/oocyte cDNA library to produce clones covering the full-length of β-1,3 glucanase (Fig. 1). Both strands of two overlapping cDNAs were sequenced, and the nucleotide and predicted amino acid sequences are shown (Fig. 2). A 50-nt 5′ upstream leader contains a consensus Kozak site and similarity to a site found in maternal mRNAs that are translated after fertilization (21, 22). The start methionine is followed by a hydrophobic stretch of 20 aa that is presumed to be the signal sequence (shaded box). This stretch terminates with a glutamine, which is the presumed V8 protease cleavage site, and is followed by a sequence that is identical to another of the peptide sequences established earlier from the native protein. All three amino acid sequences that were derived by peptide sequencing were thus found in the predicted amino acid

Strongylocentrotus purp.	U49711	IQSARLRTVESFSFKYGRLEVEAK	WPASGEIDLVESRGNAD
Tachypleus tridentatus	pir|A49878	YTSARLKTQFDKSWKYGKIEAKMA	WPSSGEIDFIEHRNTNN
Bacillus circulans	sp|P23903	YSSGKINTKDKLSLKYGRVDFRAK	WAASGEIDVMEARGRLP
Rhodothermus marinus	pir|S48201	YTSARLVTRGKASWTYGRFEIRAR	WPDNGEIDIMEHVGFNP
Sacharomyces cerevisiae 1	pir|A41624	YRSGMLQSWNKVCFTQGALEISAN	GRGAPEIDVLEGETDTK
Alteromonas carrag.	gi|437974	LYYTSGVAKSRATGNYGYYEARIK	DVQYSEIDVVELTQKSA
CORN	gi|563235	DRSSGSGFQSKAQYLYGRFDMQLK	GSQHDEIDFEFLGNASG
TOMATO	pir|D49539	DKISGSGFQSKNEYLFGRFDMQLK	GTTWDEIDFEFLGNSSG
ARAB	gi|469484	DKSSGSGFQSKNEYLFGKVSMQMK	GAGHDEIDFEFLGNSSG
SOY	pir|B49539	DKVSGSGFKSKKEYLFGRIDMQLK	GPTHDEIDFEFLGNLSG
WHEAT	gp|L43094	DKTTGTGFQTRGSYLFGHFSMHIK	NSEHDEIDFEFLGNRTG
Sacharomyces cerevisiae 2	gi|544518	KKTTGSLITSTRSFLYGKASVRMK	SAIGDEIDFEWLGGDLM
Bacillus polymyxa	gp|X57094	NKFDCGEYRSTNNYGYGLYEVSMK	GTQWDEIDIEFLGKDTT
Clostridium thermocellum	gp|X58392	YPYKSGEYRTKSFFGYGYYEVRMK	NNPWDEIDIEFLGKDTT
Rhumococcus flavefaciens	gp|S61204	PRYSGGEFRTNNFYHYGYYECSMQ	DNPWDEIDIEILGKNTT
Bacillus sp.	pir|S32688	PPYKAGELRTNDFYHYGLFEVSMK	NDPWDEIDIEFLGKDTT

FIG. 4. Alignment from two highly conserved regions totalling 41 aa for representative taxa with significant homology to *Strongylocentrotus* glucanase. This alignment was used to produce the phylogeny shown in Fig. 5.

sequence of the cDNA, each, after the first, was downstream of a glutamine (underlined). The mature protein is 499 aa, with a predicted M_r of 57 and pI of 4.1. The amino acid composition analysis made from purified enzyme in a previous report agrees well with the predicted amino acid sequence established here (14, 16).

Phylogenetic Analysis of Related β-1,3 Glucanases. We aligned the sea urchin glucanase with homologous regions of the three genes identified as its closest relatives (Fig. 3). This alignment was generated using CLUSTAL V (20). On further analysis, two highly conserved regions totalling 41 aa in length (Fig. 3, underlined) appear to be homologous to a number of other fungal, plant, and bacterial genes (Fig. 4). The results of a phylogenetic analysis using unordered parsimony is shown in Fig. 5 [phylogenetic analysis was performed using PAUP 3.1.1 (24); tree drawn by MACCLADE 3.04 (20)]. A tree generated by protpars stepmatrix (25) was not substantially different than the tree shown. The relationships at the deepest nodes of this tree should not be considered definitive. According to this tree, a yeast β-glucan gene is the sister group to the group composed of the urchin glucanase and its relatives. This result is also supported by the results of our BLASTP search (described above in Fig. 3). This search found a total of six regions of close homology between the urchin and the yeast protein, many more than the next contender, the *Alteromonas carraghenase*.

β-1,3 Glucanase Message Persists During Early Development in the Sea Urchin. A Northern blot using poly(A)$^+$ RNA was probed with the 800-bp PCR product generated as above. A 3.2-kb message was identified in ovary, oocytes, and through 19.5 hr of development (Fig. 6). This probe also recognizes a message of 3.2 kb present in eggs and of the Atlantic coast urchin *L. variegatus*. There is also a slightly larger RNA recognized in the adult gut of *L. variegatus*. A ubiquitin message was probed as a loading control. Longer exposures, sufficient to elevate the ubiquitin signal to saturating levels, failed to detect a signal to β-glucanase in the 25- and 32-hr lanes.

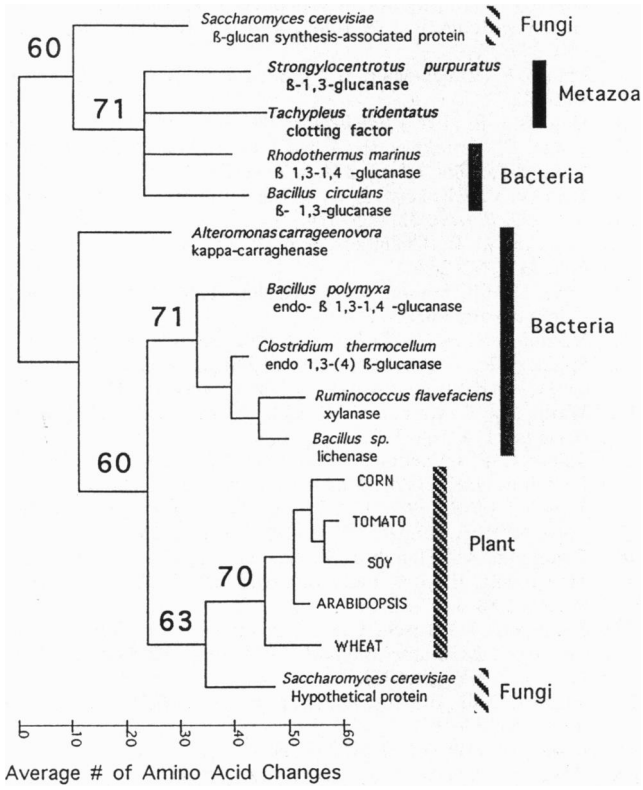

FIG. 5. Parsimony analysis of 17 glucanases and their relatives. The numbers at the major nodes are the percent of the time a node is supported in 500 bootstrap pseudoreplicates using PAUP 3.1 (24). All nodes with less than 50% bootstrap support were collapsed. The scale shown at the bottom is number of amino acid substitutions along a given branch as calculated by MACCLADE 3.0 (25).

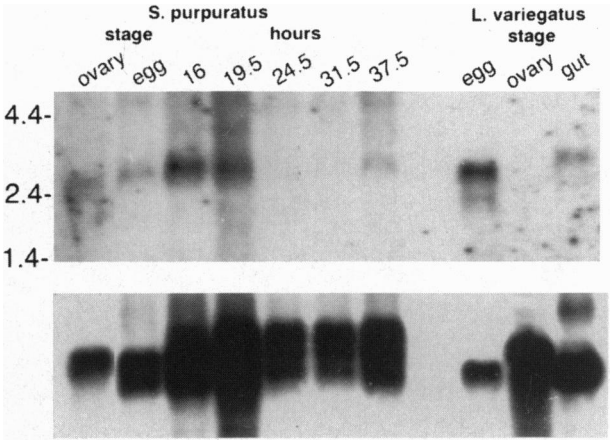

FIG. 6. Northern blot analysis of sea urchin β-1,3 glucanase. Poly(A)$^+$ RNA from the various time points was run on 1.2% formaldehyde gel and analyzed by Northern blot using the 800 bp PCR product. Dev. times for *S. purpuratus* are indicated in hours of development and correspond approximately to egg (0 hr) through gastrula (37.5 hr) stages of development. Three stages from the species *L. variegatus* are included on the same blot as indicated. A ubiquitin control was performed on the same blot, and is indicated below.

Antibody Identification of the Enzyme and the Cloned Product. The β-1,3 glucanase protein was followed during early sea urchin development by Western blot analysis using a polyclonal antibody made to the native enzyme. As shown in Fig. 7*A*, a single 68-kDa protein is present during early embryogenesis in *L. variegatus*. The enzyme is exocytosed at fertilization (Fig. 7*A*) and it persists in the perivitelline space until hatching (data not shown). By 72 hr, the embryos are actively feeding and the enzyme reappears in the gut as revealed by immunofluorescence and by Western blot analyses (Fig. 7*A*, pluteus and 2-week lanes). The antibody to native β-1,3 glucanase also recognizes the fusion protein expressed from near full-length β-glucanase cDNA, an observation that further confirms our conclusion that the gene cloned is β-1,3 glucanase (Fig. 7*B*).

DISCUSSION

This is the first molecular description of a β-1,3 glucanase in the animal kingdom. Among the numerous β-1,3 glucanases that have been cloned and described among plants, fungi, and bacteria, the closest amino acid sequence similarity exists between the sea urchin enzyme and a bacterial glucanase. These enzymes are homologous based on strong statistically supported stretches of identical amino acid sequences, especially in the active site region of the enzyme. This homology with bacteria, and little similarity to β-1,3 glucanase in other eukaryotes including those in plants or fungi, raised an obvious possibility that sea urchin β-1,3 glucanase may have arisen by horizontal transfer from a species similar to *B. circulans*. However, a more likely explanation from the phylogenetic analysis is that there are extremely ancient divergences here represented. Fig. 5 illustrates that these divergences must have occurred quite early in the prokaryotic/eukaryotic separation. However, a broader search for metazoan glucanases is necessary to distinguish between these hypotheses. Occasional biochemical reports of β-1,3 glucanases have been reported in metazoans other than echinoderms (8, 26). However, these reports are inconclusive, because in those studies whole organisms were homogenized and assayed for activity for β-1,3

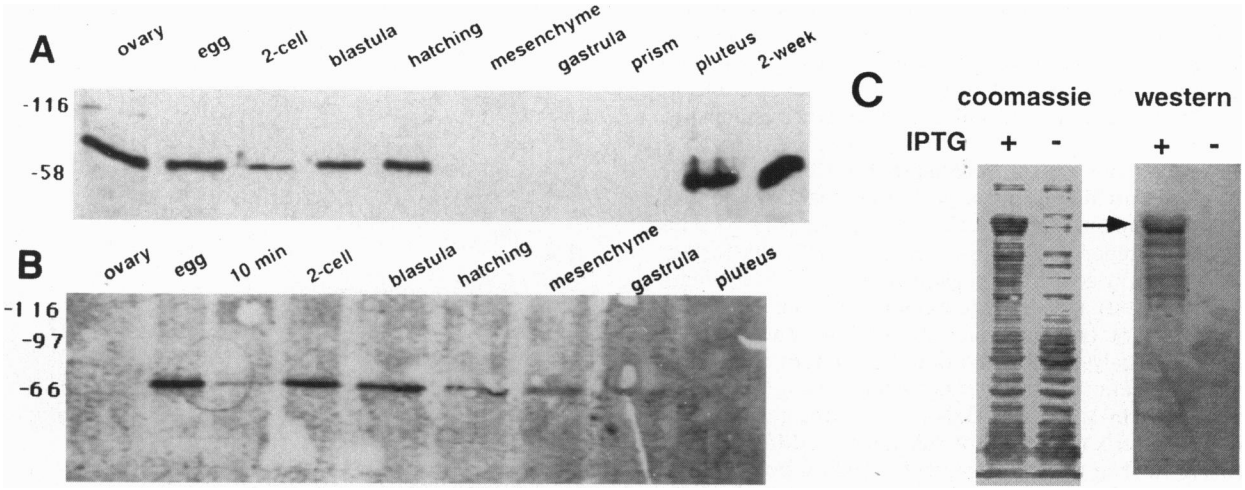

FIG. 7. Native β-1,3 glucanase analyzed by Western blot of proteins at advancing developmental stages in *L. variegatus* (*A*) and *S. purpuratus* (*B*), respectively. Molecular mass markers are indicated. (*C*) Coomassie blue stain and Western blot analysis of a 58-kDa fusion protein from a full-length sea urchin β-1,3 glucanase cDNA in a bacterial expression vector. Comparison of the induced (IPTG) versus the noninduced state is shown.

glucanase, making it likely that gut microfauna contaminated the preparations. Therefore the only known β-1,3 glucanases in the metazoa, at present, are in the Echinodermata. Two lectins, one in the horseshoe crab and the other in yeast, appear to also be homologous with the sea urchin protein.

Numerous biochemical features of β-1,3 glucanases from bacteria and sea urchins also are similar, and these activities further distinguish enzymes in the upper branch of Fig. 5 from plant and fungal β-1,3 glucanases. Enzyme characterization experiments show that sea urchin egg β-1,3 glucanase: (*i*) digests yeast cell walls, (*ii*) digests laminarin as an endoglucanase and (*iii*) does not have activity against fungi that are pathogenic for plants and that are exquisitely sensitive to plant β-glucanases (27). The *in vitro* activity of sea urchin glucanase is therefore similar to bacterial glucanase activities, and not similar to plant defense β-1,3 glucanase activities. In addition, temperature optima and the molecular size is also similar to bacterial enzymes. The optimal activity for β-glucanase in *B. circulans* and *R. marinus* is 70°C and 85°C, respectively, whereas the sea urchin form is most active at 60°C (unusual for a species that lives at 15°C and below).

What role does sea urchin egg β-1,3 glucanase play during early embryogenesis? Evidence for a glucan substrate within the fertilization/extraembryonic envelope exists in one species of sea urchin that uses a 75-kDa β-1,3 glucanase as a hatching enzyme (11). It is therefore possible that eggs and early embryos secrete β-1,3 glucanase to breakdown extracellular matrix during development, perhaps in a similar autolytic cycle seen in fungal cell wall development (28, 29). Another possibility we have tested and ruled out is that this enzyme is used by freshly fertilized embryos against marine fungal pathogens that contain β-glucans within their cell walls. Therefore we favor the hypothesis that there is an endogenous β-glucan substrate within the fertilization envelope of sea urchins. Preliminary studies suggest this to be the case, but we are pursuing this to identify the natural substrate that may provide further insight on the unusual presence of β-1,3 glucanase in the perivitelline space after fertilization.

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1. Molina, M., Cenamor, R., Sanchez, M. & Nombela, C. (1989) *J. Gen. Microbiol.* **135,** 309–314.
2. Sakellaris, H., Pemberton, J. M. & Manners, J. M. (1990) *Appl. Environ. Microbiol.* **56,** 3204–3208.
3. Simmons, C. R., Litts, J. C., Huang, N. & Rodriguez, R. L. (1992) *Plant Mol. Biol.* **18,** 33–45.
4. Ward, E. R., Payne, G. B., Moyer, M. B., Shericca, C. W., Dincher, S. S., Sharkey, K. C., Beck, J. J., Taylor, H. T., Ahl-Goy, P., Meins, F. J. & Ryals, J. A. (1991) *Plant Physiol.* **96,** 390–397.
5. Mauch, F., Mauch-Mani, B. & Boller, T. (1988) *Plant Physiol.* **88,** 936–942.
6. Henrissat, B. (1991) *Biochem. J.* **280,** 309–316.
7. Kollar, R., Petrakova, E., Ashwell, G., Robbins, P. W. & Cabib, E. (1995) *J. Biol. Chem.* **270,** 1170–1178.
8. Sova, V. V., Elyakova, L. A. & Vaskovsky, V. E. (1969) *Comp. Biochem. Physiol.* **32,** 465–474.
9. Truschel, M. R., Chambers, S. A. & McClay, D. R. (1986) *Dev. Biol.* **117,** 277–285.
10. Epel, D., Muchmore, A. V., Weaver, A. M. & Schimke, R. T. (1969) *Science* **163,** 294–296.
11. Vacquier, V. D. (1975) *Exp. Cell Res.* **93,** 202–206.
12. Spillaert, R., Hreggvidsson, G. O., Kristjansson, J. K., Eggertsson, G. & Palsdottir, A. (1994) *Eur. J. Biochem.* **224,** 923–930.
13. Watanabe, T., Kasahara, N., Aida, K. & Tanaka, H. (1992) *J. Bacteriol.* **174,** 186–190.
14. Talbot, C. F. & Vacquier, V. D. (1982) *J. Biol. Chem.* **25,** 742–746.
15. Laemmli, U. K. (1970) *Nature (London)* **227,** 680–685.
16. Truschel, M. R. (1986) Ph.D. thesis (Duke Univ., Durham, NC).
17. Foltz, K. R. & Lennarz, W. J. (1993) *Science* **259,** 1421–1425.
18. Strathman, M., Hamilton, B. A., Mayeda, C. A., Simon, M. I., Meyerowitz, E. M. & Palazzolo, M. J. (1991) *Proc. Natl. Acad. Sci. USA* **88,** 1247–1250.
19. Sambrook, J., Fritsch, E. F. & Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual* (Cold Spring Harbor Lab. Press, Plainview, NY), 2nd Ed.
20. Higgins, D. G., Bleasby, A. J. & Fuchs, R. (1992) *Comput. Appl. Biosci.* **8,** 189–191.
21. Kozak, M. (1986) *Cell* **44,** 283–292.
22. Rosenthal, E. (1993) *Dev. Genet.* **14,** 485–491.
23. Altschul, S. F., Gish, W., Miller, W., Myers, E. W. & Lipman, D. J. (1990) *J. Mol. Biol.* **215,** 403–410.
24. Swofford, D. L. (1993) PAUP, Phylogenetic Analysis Using Parsimony (Illinois Nat. Hist. Survey, Champagne).
25. Maddison, W. P. & Maddison, R. (1992) MACCLADE, Analysis of Phylogeny and Character Evolution (Sinauer, Sunderland, MA), version 3.0.

26. Turkiewicz, M., Kalinowska, H. & Galas, E. (1990) *Acta Biochem. Polonica* **38,** 79–85.
27. Planas, A., Juncosa, M., Lloberas, J. & Querol, E. (1992) *FEBS Lett.* **308,** 141–145.
28. Keitel, T., Simon, O., Borriss, T. & Heinemann, U. (1993) *Proc. Natl. Acad. Sci. USA* **90,** 5287–8291.
29. Wessel, G. M., Truschel, M. R., Chambers, S. A. & McClay, D. R. (1987) *Gamete Res.* **18,** 339–348.