Characterization of Mannuronan C-5-Epimerase Genes from the Brown Alga Laminaria digitata

Pi Nyvall, Erwan Corre, Claire Boisset, Tristan Barbeyron, Sylvie Rousvoal, Delphine Scornet, Bernard Kloareg, and Catherine Boyen*

Unité Mixte de Recherche 1931, Centre National de la Recherche Scientifique and Laboratoires Goëmar, Station Biologique de Roscoff, BP 74, 29682 Roscoff cedex, Brittany, France

Alginate is an industrially important polysaccharide obtained commercially by harvesting brown algae. The final step in alginate biosynthesis, the epimerization of $\beta$-1,4-d-mannuronic acid to $\alpha$-1,4-l-guluronic acid, a structural change that controls the physicochemical properties of the alginate, is catalyzed by the enzyme mannuronan C-5-epimerase. Six different cDNAs with homology to bacterial mannuronan C-5-epimerases were isolated from the brown alga Laminaria digitata (Phaeophyceae). Hydrophobic cluster analysis indicated that the proteins encoded by the L. digitata sequences have important structural similarities to the bacterial mannuronan C-5-epimerases, including conservation of the catalytic site. The expression of the C-5-epimerase genes was examined by northern-blot analysis and reverse transcriptase-polymerase chain reaction, and activity measurements. From both the structural comparisons and the expression pattern, it appears that the cDNAs isolated from L. digitata encode functional mannuronan C-5-epimerases. The phylogenetic relationships of the bacterial and brown algal enzymes and the inferences on the origin of alginate biosynthetic machinery are discussed.

Kelps are photosynthetic organisms that are abundant on rocky coasts from temperate to sub-Antarctic and Arctic seas. They are members of the class Phaeophyceae in the division Heterokontophyta, a phylum that emerged as an independent lineage contemporarily with the radiation of other higher eukaryote lineages (Baldauf et al., 2000). They have a haplodiplophasic life cycle, alternating between a microscopic gametophyte phase and a macroscopic sporophyte phase. Although the gametophyte phase consists of uniseriate filaments, a few millimeters long, the sporophytes have distinct organs, i.e. holdfast, stipe, and blade, and can reach from several to 50 m in length depending on the species. The kelp sporophytes are an economically important marine crop, cultivated in East Asia and harvested from natural populations in Europe and North America, including for the production of alginate, a gel-forming polysaccharide used in a large variety of pharmaceutical, food, and industrial applications (Onsøyen, 1996).

Alginate is the major matrix component of brown algal cell walls, up to 45% in dry weight (Kloareg and Quatrano, 1988). It consists of an unbranched polysaccharide made of $\beta$-1,4-d-mannuronic acid (M) and its C-5-epimer $\alpha$-1,4-l-guluronic acid (G), which are arranged in homopolymeric regions of M and G blocks, interspaced with regions of alternating structure (MG blocks). These features vary according to the nature and the age of the tissues as well as to the season and to the growing area (sheltered or exposed). The stipe of Laminaria spp., for example, contains a high fraction of G blocks, whereas the blade contains a higher proportion of M residues (Haug et al., 1974). The biological significance of this disparity is linked to the physicochemical properties of alginate, which depend on both the overall M to G ratio and the relative proportions of the three block types in the chain. A G block-rich alginate will form a high-strength gel in presence of divalent cations such as calcium, providing an increased rigidity to the stipes and holdfasts of the algae. On the other hand, alginites rich in M or MG blocks are more flexible polymers, such as required in blades exposed to wave action (for review, see Kloareg and Quatrano, 1988).

The biosynthetic pathway of alginate in brown algae is shown in Figure 1 (Lin and Hassid, 1966; Hellebust and Haug, 1969; Madgwick et al., 1973). The final step is epimerization of $\alpha$-mannuronic residues into l-guluronic residues within the polymer chain, a reaction catalyzed by mannuronan C-5-epimerases. Given their chemical structure and biological function, i.e. to provide both strength and flexibility to the algal tissue, alginites may be thought of as the functional analogues of the pectins of higher plants. It follows that mannuronan C-5-epimerases probably are functionally analogous to the plant pectin methylesterases (PMEs) in control-
ling the texture of the cell wall matrix. Guluronate-rich alginate is also present in calcareous red algae, where it is involved with the deposition of calcium carbonate (Okasaki et al., 1982, 1984; Usov et al., 1995). Alginate constitutes also the sheath of some bacteria, namely *Pseudomonas* spp. and *Azotobacter vinelandii*, and most of the current knowledge on the genes involved in alginate biosynthesis comes from bacterial systems (Rehm and Valla, 1997). Two different types of mannuronan C-5-epimerase genes are featured in *A. vinelandii*, the modular AlgE genes, which form a multigene family with seven members, and the non-modular AlgG, which was found only as a single-copy gene (Rehm et al., 1996). This latter gene is localized within the cluster of genes involved in the alginate biosynthesis pathway, whereas the AlgE genes form a separate cluster (Rehm and Valla, 1997). In contrast to alginate-producing bacteria, no enzyme in the biosynthetic pathway of alginates, including mannuronan C-5-epimerase, was purified or cloned from brown algae.

We recently carried out an expressed sequence tag (EST) analysis from the sporophytes and gametophytes of the kelp *Laminaria digitata* (Crépineau et al., 2000). A total of 905 ESTs was established from both life history stages, leading to the characterization of approximately 600 different transcripts. They included six cDNAs involved in carbon metabolism, the expression of which was analyzed in detail (Moulin et al., 1999). In addition, two ESTs were shown to exhibit significant similarity with the coat protein GP1 from a virus (EsV-1) infecting the filamentous brown alga *Ectocarpus siliculosus*. To get more information on the putative function of these genes, the corresponding cDNAs (hereafter referred to as ManC5-E1 and ManC5-E2) were fully sequenced, thus revealing a significant similarity to the mannuronan C-5-epimerases from *A. vinelandii* and *Pseudomonas aeruginosa*. We report here the sequence characterization, expression pattern, and phylogenetic analysis of six mannuronan C-5-epimerase genes from *L. digitata* and discuss their evolutionary relationships with the bacterial and viral genes.

**RESULTS**

*L. digitata* Harbors a Variety of Mannuronan C-5-Epimerase Genes

The coding region of the ManC5-E1 cDNA was used as a homologous probe to screen a cDNA library of *L. digitata* sporophytes, leading to the identification of four new cDNAs encoding mannuronan C-5-epimerase. The cDNAs were fully sequenced and are referred to as ManC5-E3 to ManC5-E6. As illustrated in Figure 2, alignments of the deduced amino acid sequences and of the nucleotide sequences indicated that two (ManC5-E1 and ManC5-E6) of the six
different cDNAs were full length, whereas the others were partial cDNAs. They all contained large 3'-UTRs varying in length between 1,318 and 1,825 bp. The two full-length cDNAs featured ORFs encoding for proteins of 55 kD, with no conspicuous glycosylation sites, and they both contained short 5'-UTRs. The six cDNAs shared a high level of identity (64% and 59%, based on the nucleotide and protein sequences, respectively) over the 802 bp (267 amino acids) of the coding region that was available for all six clones, whereas the 3'- and 5'-ends were very variable.

Using a 1-kb fragment of the coding region of ManC5-E1 as a probe (Fig. 2), eight different genomic clones of mannuronan C-5-epimerases, referred to as EpiA to H, were identified by screening a genomic library from *L. digitata*. Hybridization experiments with specific 3'-UTR probes from each of the six cDNAs indicated that only one genomic clone, EpiG, matched with one of the cDNAs, ManC5-E6. Thus, the EpiG clone was mapped, subcloned, and sequenced. The DNA fragment encompassed an entire mannuronan C-5-epimerase ORF, i.e. from before the start codon to the end of the 3'-UTR (Fig. 3). However, the EpiG ORF displayed only 89% nucleotide identity to ManC5-E6. The 3'-UTR part was even less conserved, with an identity of 57% within the region used as a probe (55% over the whole 3'-UTR). Based on sequence comparison between EpiG and the cDNA clones, six introns, ranging in size from 295 to 513 bp, were located in the ORF (Fig. 3). All, except one, of the exon/intron/exon transitions were marked by the consensus sequence of universal splice sites for nuclear-encoded eukaryotic genes, G GTXXG . . . .AG C/G (Breathnach and Chambon, 1981; Villand et al., 1997). The presence of a variety of mannuronan C-5-epimerase genes in the *L. digitata* genome was confirmed by Southern-blot analyses, where a large number of bands were detected (Fig. 4).

**Phylogenetic Relationships of Mannuronan C-5-Epimerases**

The ManC5-E1 sequence from *L. digitata* was subjected to similarity search against the NCBI non-redundant protein database using the BLASTX software, revealing that the most similar sequences were the coat proteins GP-1 from *E. siliculosus* virus and from *Ectocarpus fasciculatus* unclassified viruses. Scores obtained were around 45% of identity and 65% of similarity based on sequence data. ManC5-E1 also matched the non-modular C-5-epimerases AlgG from *A. vinelandii* and *P. aeruginosa* and AlgE1–7 and AlgY from *A. vinelandii* (20%–27% identity), mainly due to a conserved motif from Tyr-278 to Asp-284 (numbering of ManC5-E1 sequence).

The similarity between these proteins was investigated further by hydrophobic cluster analysis (HCA) of the amino acid sequence of ManC5-E1, together with those of the GP-1 coat protein from the *E. siliculosus* virus, AlgG from *P. aeruginosa*, and the module A of AlgE5 epimerase from *A. vinelandii* (Fig. 5).
As expected from BLAST results, the *L. digitata* sequence showed the highest structural similarity to the GP1 viral protein, although over a shorter domain (from Lys-150 to Phe-373). As shown by the occurrence in all sequences of several strictly conserved hydrophilic residues (negatively colored in Fig. 5), ManC5-E1 (from Leu-66 to Leu-332) also displayed a high structural similarity to AlgG, notably over a domain of 133 amino acids (from Met-199 to Leu-332).

The deduced amino acid sequences of the six ManC5-E from *L. digitata* were aligned with those of bacterial epimerases and of the viral capsid protein GP-1 (alignment available upon request). A phylogenetic tree was built by neighbor-joining analysis using the Dayhoff distance matrix (Fig. 6). Thus, a tree based on parsimony presented the same topology and the corresponding bootstrap values are also included in the tree. The ManC5-E proteins of *L. digitata* form a monophyletic group, which includes the EsV-1 protein GP1, and with the AlgG sequences from *Azotobacter* spp. and *Pseudomonas* spp. as the closest cluster. Within the *L. digitata* cluster, ManC5-E proteins form three different subgroups with one, comprising ManC5-E1, E2, and E4, supported by a high bootstrap value (99%). Most distantly related to the epimerases from *L. digitata* is the well-supported cluster of modular epimerases from *A. vinelandii*, known as AlgE and AlgY.

**Mannuronan C-5-Epimerase Transcripts in *L. digitata***

The expression of the C-5-epimerase genes was examined by northern-blot analysis and reverse transcriptase polymerase chain reaction (RT-PCR) in sporophytes collected at regular intervals over a whole year. Based on hybridization with the cDNA probe (Fig. 2), one single approximately 3.5-kb transcript was observed (Fig. 7). However, cloning and sequencing of the RT-PCR products indicated that as many as 16 different epimerase genes were expressed in sporophytes (data not shown). Both northern-blot and RT-PCR analyses showed that the transcript level depended on the period of the year: Only a weak expression of epimerase was observed in September and November; the expression increased in January, remained high in February, then decreased slightly, but remained higher than in the autumn samples until April (Fig. 7); no transcript could be detected in sporophytes collected from May to August (data not shown).

The expression of C-5-epimerase genes was then analyzed in protoplasts from young sporophytes of *L. digitata*. Based on the [5-3H] poly-mannuronate assay (Rehm et al., 1996), most of the epimerase activity was present in the protoplast culture medium, and only a low level could be detected in the protoplasts themselves (data not shown). Epimerase activity in the culture medium was highest in the 1st...
of protoplast culture (Fig. 8A). A similar trend was observed in the level of epimerase transcripts, as observed by northern blot or RT-PCR (Fig. 8B). A faint but significant signal, corresponding to an apparent molecular mass of approximately 75 kD, was observed upon immunoblotting the protoplast culture medium with an antiserum directed against ManC5-E6 (Fig. 8C). This apparent molecular mass is higher than that predicted from the sequence, 55 kD. A similar size discrepancy was observed for the EsV-1 GP1 protein (Klein et al., 1995) and for the epimerases from A. vinelandii (Ertesvåg et al., 1994, 1998; Ertesvåg and Valla, 1999).

DISCUSSION
Mannuronan C-5-Epimerases Are Encoded by a Gene Family in L. digitata

Here, we have isolated six different cDNAs from the sporophytes of the brown alga L. digitata, which exhibit significant similarities with mannuronan C-5-epimerase genes from alginate-producing bacteria and with a coat protein gene from a virus infecting the brown alga E. siliculosus. In particular, all these genes share a conserved motif, Tyr-Gly-Phe/Iso-Asp-Pro-His-Asp/Glu (Fig. 9). Based on site-directed mutagenesis of the first Asp residue into a Gly residue, this motif was shown to be the catalytic site of module A1 in AlgE1 epimerase (Svanem et al., 2001). Our attempts (using either Escherichia coli or Pichia pastoris and ManC5-E1 and ManC5-E6) to produce the mannuronan C5-epimerase of L. digitata in an active form were not successful (data not shown). Due to a variety of technical difficulties, purification of mannuronan-5′-epimerase from protoplast isolation medium was not considered feasible. Nevertheless, based on the sequence similarities with the bacterial mannuronan C-5-epimerases, including the presence of the catalytic site (Fig. 9), and on the occurrence of conserved secondary structure elements in all of these proteins (Fig. 5), we propose that the ManC5-E genes of L. digitata encode functional mannuronan C-5-epimerases, the enzymes involved in the last step of the biosynthesis of alginic acid (Fig. 1). As discussed below, this proposition is consistent with the pattern of expression of the ManC5-E genes in L. digitata.

In addition, we have identified eight different genomic clones that hybridize with the ManC5-E probe. None of them, however, completely matched with any of the six cDNA clones. Also, sequence analysis of the ManC5-E amplification products, corresponding to a 400 bp region within the ORF (Fig. 2), unraveled at least 16 distinct transcripts in L. digitata sporophytes, of which only one perfectly matched one of the cDNAs, ManC5-E2. Despite the absence of detailed sequence information on most of the genomic clones, these observations indicate that...
L. digitata features at least 21 different mannuronan C-5-epimerase genes. An additional diversity may occur due to the presence of long 3'-UTRs in the L. digitata ManC5-E genes. Consistently, Southern-blot analysis indicated the presence of a large family of ManC5-E genes in L. digitata (Fig. 4).

Expression of Mannuronan C-5-Epimerases in L. digitata

Based on northern-blot and RT-PCR analyses, the expression of ManC5-E genes was maximal in winter and early spring (Fig. 7). This corresponds to the period when seawater nutrient levels are the highest in Brittany and to the time of the most active growth and the largest increase in the alginate acid content of L. digitata (Pérez, 1971). Because naked, wall-less cells, protoplasts turn their metabolism toward the building of a new cell wall, and, consistent with the requirement for alginate synthesis, epimerase activity has already been detected in the culture medium of protoplasts from L. digitata (Rødde et al., 1993). Here, we show that secretion of epimerase activity from L. digitata protoplasts correlates well with the immunological, northern, and RT-PCR observations of the expression of ManC5-E genes (Fig. 8). Interestingly, in an EST analysis of L. digitata protoplasts (V. Roeder, unpublished data), five different transcripts were found. Only two of them corresponded to those detected in normal sporophytic tissues, namely ManC5-E3 and ManC5-E6. Consistently, mannuronan C-5-epimerases were immunologically detected in protoplast culture medium using antibodies raised against peptides from ManC5-E6. Thus, it appears that the ManC5-E genes are expressed at the time of alginate biosynthesis, another piece of evidence showing that they do encode mannuronan C-5-epimerases.

The presence of a large gene family of mannuronan C-5-epimerases in L. digitata suggests that, even though they fulfill the same biochemical function, epimerization of the d-mannuronic residues of alginates into l-guluronic units, these enzymes are involved in a variety of biological functions. Depending on the season, the age of the plant, and the tissue type, different epimerases are likely to be required to specifically tailor the relative contents and distributions of G blocks, M blocks, and MG blocks in alginate chains. Consistently and as mentioned above, at least 16 distinct transcripts were detected in L. digitata sporophytes. This situation is reminiscent of that of PMEs, the functional analogues of mannuronan C-5-epimerases in higher plants. By removing methyl-ester substituents from the poly-GalUA backbone of pectins, PMEs control cell wall cross-linking, and they are implicated in many physiological processes, such as pH modulation, cell wall porosity, fruit maturation, and viral movement (for review, see Micheli, 2001). They are encoded by a multigene family, which includes as many as 67 members in Arabidopsis (Micheli, 2001).

Figure 8. Expression of mannuronan C-5-epimerase in L. digitata protoplast cultures. A, Epimerase activity in the culture medium, as measured with the [5-3H] poly-mannuronate assay. Samples were assayed in triplicates. Error bars = sds. B, Epimerase expression in protoplasts, as detected by northern blot and RT-PCR, as in Figure 7. C, Western blot of proteins from the protoplast culture medium. Proteins (2.1 μg lane −1) were separated by 12% (w/v) SDS-PAGE, transferred onto a nylon membrane, and probed with an antiserum made against two peptides of ManC5-E6 (shown in Fig. 2). Incubation of the blot with pre-immune serum did not reveal any nonspecific cross-reaction (data not shown).

Figure 9. Partial alignment of mannuronan C-5-epimerases, in the region of the catalytic domain. Strictly conserved amino acids are negatively colored. Star, Catalytic Asp residue of module A1 of AlgE1 epimerase.
Origin and Evolution of Mannuronan C-5-Epimerases

In the phylogenetic analysis of ManC5-E genes (Fig. 6), the mannuronan C-5-epimerases of L. digitata form a monophyletic group, which shares a close common ancestor with the GP1 gene from the E. siliculosus virus, EsV-1. EsV-1 is a DNA virus infecting the filamentous brown alga E. siliculosus. The viral genome is integrated in the algal host DNA and transmitted in a Mendelian fashion (Müller, 1991; Bräutigam et al., 1995). The GP1 protein was first identified as the largest of the three glycoproteins that form the virus coat (Klein et al., 1995) and was recently recognized as having similarities with bacterial mannuronan C-5-epimerases (Delaroque et al., 2001). Here, we confirm that the GP1 protein is structurally related to mannuronan C-5-epimerases, and we show that it is more closely related to brown algal genes.

This raises the question of the respective origin of the viral and algal mannuronan C-5-epimerase genes. Several lines of evidence indicate that the mannuronan C-5-epimerases from L. digitata are encoded by genuine brown algal genes rather than by recently imported genes of viral origin. On the one hand, the high variety of these genes in L. digitata suggests an ancient origin. In addition, the organization of the EpiG gene, with several introns that exhibit consensus splicing motifs (Fig. 3), is typically eukaryotic in nature, and the ManC5-E cDNAs feature long 3′-UTRs (Fig. 2), a common characteristic of L. digitata genes (Apt et al., 1995; Crépineau et al., 2000). On the other hand, the complete sequence of EsV-1 genome (Delaroque et al., 2001) shows the presence of only four genes that could be involved with alginate biosynthesis, namely the capsid protein GP1, a UDP-Glc/GDP-Man dehydrogenase and, possibly, the ORF 83 and ORF 84 proteins. Altogether, and because viruses are known to acquire prokaryotic or eukaryotic DNA elements (van Etten and Meints, 1997; Huang et al., 1999; Michel et al., 2001; Rigden and Franco, 2002). These observations suggest that this helical cap motif is a signature of solenoid-type fold, where the α-helix forms a strand-helix-strand cap that covers the N-terminal end of the solenoid (Steinbacher et al., 1994; Lietzke et al., 1996; Petersen et al., 1997; Huang et al., 1999; Michel et al., 2001; Rigden and Franco, 2002). These observations suggest that the filamentous cyanobacterium, which then evolved into red or green plastids (Douglas, 1998; Lang et al., 1999; Moreira et al., 2000). The Phaeophyceae are thought to have arisen from a secondary endosymbiosis, i.e. between a plastid-less protist and an ancestral, unicellular red alga (Baldauf et al., 2000). Eukaryotic cells are considered genetic chimera; the result of multiple lateral transfers from a variety of different organisms (Sitte, 1993). Based on the relatedness of the cellulose synthase genes of Cyanobacteria with those of vascular plants, it is assumed that plants have acquired cellulose synthases through the plastid endosymbiosis (Nobles et al., 2001). To account for the relatedness of bacterial and algal mannuronan C-5-epimerases, it is tempting to envision a similar scenario, as follows. Mannuronan C-5-epimerase—and probably the whole metabolic
pathway for the biogenesis of alginate—which would have arisen in ancient bacteria (of which A. vinelandii and P. aeruginosa are extant relatives). A gene of the AlgG type could have been acquired via the plastid primary symbiosis by red algae (where it may still be retained in the family of Corallinaceae; Okasaki et al., 1982, 1984; Usow et al., 1995) and then would have been passed on to brown algae via the secondary endosymbiosis, where it diverged into a multigene family.

However, because there are no alginate-biosynthetic genes in extant cyanobacteria (http://w3.integrated-genomics.com/GOLD), the algal biosynthetic pathway could alternatively have been the result of lateral transfer from a photosynthetic or non-photosynthetic prokaryote. In any case, we view the acquisition of a mannuronic C-5-epimerase gene by the EsV-1 virus as posterior to the emergence of brown algae.

In conclusion, the structural similarities of L. digitata ManC5-E genes, including at the three-dimensional structure level, to functional bacterial mannuronic C-5-epimerases together with the fact that their expression correlates well with alginate biosynthesis validate the identity of these sequences as mannuronic C-5-epimerases. This is the first characterization, to our knowledge, of a gene involved in the synthesis of alginates in brown algae, and this finding is of primary interest for further understanding cell wall assembly and function in these marine plants. It also sheds some light on the origin and evolution of the alginate biosynthetic pathway. Finally, brown algal mannuronic C-5-epimerases may prove interesting tools for engineering alginate functional properties. However, this will require the expression of active enzymes, an objective that was not attained in this study. It is perhaps worth noting here that, to our knowledge, it has not yet been possible either to overexpress higher plant PMEs, a failure that is accounted for by the occurrence in these enzymes of specific posttranslational processing constraints (Micheli et al., 1998).

MATERIALS AND METHODS

Identification of Mannuronic C-5-Epimerase Clones

Two cDNA clones with similarity to mannuronic C-5-epimerases (ManC5-E1 and ManC5-E2) were identified from a data set of 905 ESTs made from two oriented cDNA libraries of sporophytes and gametophytes of Laminaria digitata L. Lamarck (Crépinoiu et al., 2000). Screening of a sporophyte cDNA library with ManC5-E1 led to the identification of an additional four cDNA clones (ManC5-E3 to ManC5-E6). The ManC5-E nucleotide sequence data are deposited in the EMBL database under accession numbers AJ496449 to 54. Genomic DNA from L. digitata sporophytes was isolated according to Apt et al. (1995) and partially digested with Sau3A. DNA fragments between 8 and 12 kb were ligated into BamHI-digested Lambda Dash II vector (Stratagene, La Jolla, CA). The vector was packaged using Gigapack III Gold Packaging extract (Stratagene), resulting after amplification, in a genomic library of 56 × 10^6 plaque forming units. Using a 1-kb fragment from ManC5-E1 (Fig. 2), eight different genomic clones, referred to as EpiA to H, were isolated from this library.

Sequence Comparisons

The amino acid sequences of the L. digitata epimerase clones were aligned manually with a representative set of amino acid sequences obtained from recent GenBank releases (Benson et al., 1999) using the Seaview software (Galtier et al., 1996). HCA alignments (see below) were used as a guide to ensure that only homologous regions were compared, amounting to a total of 107 amino acid informative positions. Distance matrix, maximum parsimony methods (Fitch, 1971) were applied, as recommended in using the Phylo-Win software package (Galtier et al., 1996). The neighbor-joining method (Saitou and Nei, 1987) was performed with several distance correction factors, and bootstrap analysis was used to provide confidence estimates for the phylogenetic tree topologies (Felsenstein, 1985). HCA (Callebaut et al., 1997) was used to compare at the secondary structure level the amino acid sequences of ManC5-E1 (GenBank accession no. AJ496449), AlgE5 from Azotobacter vinelandii (TreMBL no. Q44492), AlgG from Pseudomonas aeruginosa (TreMBL no. Q51371), and the GP-1 protein from the Ectocarpus virus (TreMBL no. Q8QN64).

Gene Expression Analyses

Total RNA was isolated from protoplasts and from sporophytes of L. digitata collected at Île de Sieck (Brittany, France) between May 2000 and April 2001 using the protocol of Apt et al. (1995). Northern blotting was performed according to Sambrook and Russel (2001), with 20 µg of RNA per lane. Total RNA (300 ng) was used as template for PCR using the RT-PCR Beads Ready to Go kit (Amersham, Buckinghamshire, UK) according to the manufacturer’s instructions. A specific primer pair (Aliepi3, 5’ C5TGWGAAGGAGGCCCCAC5’, and Aliepi4, 5’ TCGGTGATGCTCCTCGGA 3’) was constructed against a region conserved in all six cDNA clones (Fig. 2). These primers bridge two introns in the genomic sequence, resulting in differently sized PCR fragments from mRNA and DNA amplification, 230 and 700 to 900 bp, respectively. The annealing temperature was 53°C. Two negative controls were made, consisting of addition of water to the reaction instead of RNA, and heating the reaction for 10 min at 95°C before the RT-PCR reaction.

Protoplast Isolation and Culture

Sporophytes of L. digitata, 15 to 50 cm in length, were collected at Île de Sieck in February 2002. The algae were stored in flowing seawater and used within 1 to 7 d. Immediately before use the surface of the blades were scraped with a razor blade to remove any contaminating organisms, and the meristematic areas were roughly chopped in sterile seawater and incubated for 10 min at 8°C in seawater with 1% (w/v) betadine. The betadine solution was then removed by filtration, and the algal tissue was washed and immersed for 45 min at 8°C in sterile 70% (v/v) seawater (diluted with deionized water). From this point onwards, the method of Butler et al. (1989) was used, with the modification of using artificial seawater supplemented with MgCl₂ and KCl as osmoticum during the cell wall digestion step. Protoplasts were cultivated at the cell density of 1.5 × 10⁶ cells mL⁻¹ at 12°C in plastic petri dishes, 9 cm in diameter, containing 10 mL of sterile seawater enriched with osmotomuc, nutrients, rifampicine (0.4 µg mL⁻¹) and carbenicilin (4.8 µg mL⁻¹). They were maintained in total darkness for 24 h and thereafter in dim light with a light period of 14 h of light and 10 h of darkness (Benet et al., 1997). Based on Calcofluor staining, the protoplasts had regenerated a cellulosic cell wall within 2 d of culture. Samples of protoplasts and of the culture medium were taken at intervals (0, 24, 45, 68, and 92 h), separated by centrifugation at 50g for 10 min, frozen in liquid nitrogen, and stored at −80°C. The medium was concentrated 15-fold in a stirred ultrafiltration cell (Amicon, Beverly, MA) using a YP3 membrane (Millipore, Bedford, MA) with a cutoff of 3,000 kDa before it was used in epimerase activity assays.

Mannuronic C-5-Epimerase Activity

The activity was measured with [5-3H] poly-mannuronate (1,200 KBq mg⁻¹) as substrate according to the protocol of Skjåk-Bræk et al. (1996). The poly-mannuronate, both labeled and unlabeled, was kindly provided by G. Skjåk-Bræk, Norwegian University of Science and Technology (Trondheim, Norway). In brief, reaction mixtures (final volume of 0.5 mL) consisted of
100 μL of [5-3H]-poly-mannuronate (1 mg mL\(^{-1}\) in water), up to 350 μL of cell extract or protoplast medium, and buffer to a final concentration of 20 mm MOPS (pH 7.0) and 2 mm CaCl\(_2\). Samples were incubated at 32°C for 24 to 96 h, and reactions were stopped by addition of 15 μL of 5 μ NaCl and 800 μL of isopropanol. Poly-mannuronate was precipitated by incubation at \(-80^\circ\)C for 1 h followed by centrifugation at 13,000 rpm for 35 min. The amount of radioactivity released into 1 mL of supernatant was then determined by liquid scintillation counting. To discriminate mannanuronan C-5-epimerase from alginate lyase activity (Svanem et al., 2001), the results from the above method were validated by NMR spectroscopy, as follows. The epimerization reaction was performed at 32°C for 8 d in a buffer volume of 20 mL (20 mm MOPS [pH 7.0] and 2 mm CaCl\(_2\)) containing 20 mg of poly-mannuronate and 10 mL of concentrated protoplast medium. The polysaccharide was then dialyzed against water (five changes of 5 L), lyophilized, and sent to the Norwegian University of Science and Technology, where it was analyzed by high-field 1H NMR (Grasdalen, 1983). Relative to controls, a peak with a chemical shift of 5.1 ppm and corresponding to guluronate residues (Grasdalen, 1983) had appeared on the poly-mannuronate samples incubated in the presence of protoplast culture medium (data not shown), indicating that epimerization did take place.

Western-Blotting Methods

Polyclonal antibodies (Eurogentec S.A., Herstal, Belgium) were raised against two 16-amino acid peptides (GKDTKSPNEPIPKEC and CNGRAKNNMGEARMDI) from the ManC5-E6 (Fig. 2). The peptides were coupled to hemocyanin from keyhole limpet using m-maleimidobenzoyl-N-hydroxysuccinimide ester. Two rabbits were immunized with the mixed peptides and bled after six immunizations. Serum from both rabbits was mixed and diluted 2,000-fold before use. Protein extracts were mixed with sample buffer and boiled for 5 min before application on polyacrylamide gels with a 3.9% (w/v) stacking gel and a 12% (w/v) running gel. The gels were run on a mini-protein II vertical gel electrophoresis cell (Bio-Rad, Munich, Germany) with a discontinuous Tris-Gly buffer system (Laemmli, 1970). After electrophoresis, proteins were transferred onto a nylon membrane for immunostaining (Towbin et al., 1979). Detection of the primary antibody was performed with a goat anti-rabbit antibody conjugated to horseradish peroxidase (HRP). The labeled proteins were visualized on x-ray film using the ECL kit (Amersham).

Standard Laboratory Procedures

Agarose gel electrophoresis, restriction enzyme digests and ligations were performed according to Sambrook and Russell (2001). Protein concentrations were measured using the Bio-Rad Coomassie Brilliant Blue based protein assay using bovine serum albumin as standard. DNA was sequenced using the ABI PRISM Dye Terminator Cycle Sequencing kit (Perkin-Elmer Applied Biosystems, Foster City, CA) on an ABI Prism 3100 Genetic analyzer or with the Amersham RPN 2444 cycle sequencing kit on a Vistra sequencer.

Distribution of Materials

Upon request, all novel materials described in this publication will be made available in a timely manner for noncommercial research purposes, subject to the requisite permission from any third party owners of all or parts of the material. Obtaining any permissions will be the responsibility of the requestor.

ACKNOWLEDGMENTS

We are grateful to Gurvan Michel and Philippe Potin for helpful discussions, to Gudmund Sjåk-Bræk for providing us with poly-mannuronate-rich alginate, and to Wenche Irene Strand for NMR analyses. We also thank Florence Crépineau for his contribution to the identification of the two initial C-5-epimerase ESTs.

Received April 25, 2003; returned for revision June 9, 2003; accepted July 11, 2003.


LITERATURE CITED
Mannuronan C-5-Epimerase Genes from Laminaria digitata