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Molecular analysis of the amylase gene and its expression during development in the winter flounder, *Pleuronectes americanus*

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Abstract

Determination of the onset of amylase production in marine fish larvae is difficult due to their small size and the possible presence of exogenous amylases from prey organisms in the diet or from the gut flora. In order to develop a sensitive PCR-based assay for the detection of fish-specific amylase in larvae, a complete cDNA and partial genomic sequence, the first reported from a teleost fish, were determined from winter flounder.

The complete cDNA for alpha amylase is 1539 bp and the deduced polypeptide sequence is 512 amino acids, including a putative 15 amino acid signal peptide. The molecular weight of the mature protein is 55,769 Da and the predicted isoelectric point is 6.76. Southern hybridisation analysis showed that the winter flounder amylase cDNA could be used to detect homologs in other species, particularly flatfish, and that there are likely two copies of the gene in the winter flounder genome. The winter flounder genomic sequence corresponding to amino acids 194–404 (including three introns) was amplified by the polymerase chain reaction (PCR) and the sequence used to design primers for PCR-based assays for amylase gene expression in larval and adult fish.

The levels of expression of the amylase gene from larvae sampled at 5, 13, 20, 27 and 41 days post-hatch (dph) were determined using the housekeeping gene, actin, as a control. Amylase transcripts were first detected at 5 dph, peaked at 20 dph and then decreased during metamorphosis. The amylase gene is highly expressed in adult winter flounder. This sensitive assay will be

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useful for investigating amylase gene expression under different feeding conditions and help in the development of optimal diets.

Keywords: Amylase; cDNA; Gene expression; Larvae; Reverse transcription-polymerase chain reaction (RT-PCR)

1. Introduction

Marine fish larvae require live prey for the first several weeks of their life. Although they may be weaned onto artificial feed, they seldom exhibit as good growth or survival (Person-Le Ruyet et al., 1993). Weaning is a critical phase in the life of the fish and represents one of the major challenges for aquaculture. Weaning of winter flounder has been shown to be most successful after metamorphosis (Lee and Litvak, 1996) although it was suggested that earlier weaning might improve growth. Knowledge of the timing of digestive enzyme expression in developing larvae is essential for assessing the appropriateness of formulated larval diets and the earliest possible time for weaning. In order to evaluate how well larval flatfish may be able to digest carbohydrate (a significant component of artificial diets) at different times during larval development, the cDNA for amylase was cloned from winter flounder and its expression assayed in larvae.

Alpha-amylase (α-1,4-alpha-D-glucan glucanohydrolase, EC 3.2.1.1) is responsible for the hydrolysis of α-1,4 glycoside bonds in starch and glycogen. Amylase is widely distributed in marine fish (Shimeno et al., 1977) including flatfish (Glass et al., 1987) where it is one of the major carbohydrases, and is also present in the intestinal microflora of fish (Sugita et al., 1996), although it is unclear whether this source contributes to hydrolysis of starch in fish digestive tracts. Amylase is produced by pancreatic cells located in a diffuse mesentery surrounding the digestive tract (Kurokawa and Suzuki, 1996).

Amylase activity has been detected biochemically in the digestive tract of numerous marine fish, and several studies have investigated the effect of diet on amylase expression (Cahu and Zambonino Infante, 1994; Zambonino Infante et al., 1996; Peres et al., 1998). However, there are few reports on the developmental expression of amylase in fish (Kawai and Ikeda, 1973; Sinha, 1978; Baragi and Lovell, 1986; Yardley, 1988; Moyano et al., 1996; Peres et al., 1998), especially at the molecular level. Since biochemical detection of amylase does not discriminate between enzyme produced by the fish and that present in the gut flora or prey organisms such as *Artemia* (Cousin et al., 1987), we have developed a fish-specific PCR-based assay for amylase expression.

2. Materials and methods

2.1. cDNA libraries

cDNA libraries were constructed in the λ-ZapII vector (Stratagene, La Jolla, CA, USA) from mRNA isolated from a variety of organs of the digestive tract of winter

flounder, as described by Douglas and Gallant (1998). Since pancreatic tissue is present in the diffuse mesentery surrounding the internal organs of winter flounder, the library source was not critical; in this study, a spleen library was used.

2.2. Probe production by PCR

Primers for PCR were designed based on highly conserved regions from complete amylase sequences from a number of vertebrates and invertebrates. The organisms and their GenBank accession numbers were as follows: mosquitoes *Aedes aegypti* (AF000568), *Culex tarsalis* (U01211) and *Anopheles gambiae* (L04753); fruit fly, *Drosophila melanogaster* (X04569); shrimp, *Penaeus monodon* (X77318); mouse, *Mus musculus* (V00718); and rat, *Rattus norvegicus* (M24962). Oligonucleotide primers were synthesised on an Expedite Nucleic Acid Synthesis System (PerSeptive Biosystems, Framingham, MA, USA).

Amplifications were performed using degenerate primers amy1 and amy3 (Table 1) with one million recombinant phage from the primary unamplified cDNA library as template. PCR conditions were: 30 s at 94°C; 33 cycles of 30 s at 94°C, 30 s at 30°C, 60 s at 72°C; 2 min at 72°C. Nested reactions of the primary amplification reaction were performed using amy2 and amy3. Amplification products were resolved on an agarose gel and a band of the expected size (550 bp) was obtained. These amplification products were purified using Centricon 100 columns (Amicon, Danvers, MA, USA), reamplified, resolved on an agarose gel and purified using GeneClean (Bio101, La Jolla, CA, USA). Purified products were cloned into the pCR2.1 vector (Invitrogen, Carlsbad, CA, USA) and sequenced using an ABI373 stretch automated sequencer (Perkin Elmer, Foster City, CA, USA) and the AmpliTaqFS dye terminator cycle sequencing ready reaction kit. Both strands of each recombinant were sequenced at least once.

2.3. cDNA screening

Duplicate plaque lifts from five plates each containing 50,000 recombinant plaques (pfu) from the cDNA library were hybridised with radioactively labelled PCR-derived amylase probes at 65°C for 36 h as described (Douglas et al., 1999a). Filters were washed at 65°C in 1 × SSC/0.1% SDS and positively hybridising plaques identified by autoradiography. Insert sizes were determined by PCR using a primer specific to the 5′ end of the amylase PCR product (amy5′; Table 1) in combination with T3 or T7 primers present in the multiple cloning site of the λ-ZapII vector. One plaque of the appropriate

Table 1
Oligonucleotide primers used in PCR reactions

Primer	Amino acid sequence	Nucleotide sequence (5′ > 3′)
amy1	PWWERYQP	CC[T/C]TGGTGGGAG[C/A]GNTACCAGCC
amy2	LDAAKHMWP	CTGGA[C/T]GC[C/T]GC[C/T]AAGCA[C/T]ATGTGGCC
amy3	WVCWHRW complement	CCANC[G/T][A/G]TGCTC[A/G]CA[C/G]ACCCA
amy5′	IFQEVID complement	GATCAATAACCTCCTGGAAGA

size was replated at 500 pfu/plate and screened until 100% purity of the amylase-containing recombinant was obtained.

2.4. PCR amplification of genomic sequences

Winter flounder genomic DNA (100 ng) was incubated with Taq polymerase, 10% DMSO and primers amy2 and amy3 (Table 1) in a total volume of 50 μl and subjected to the following amplification conditions: 30 s at 94°C; 30 cycles of 30 s at 94°C, 30 s at 55°C, 90 s at 72°C; 2 min at 72°C. Products (1350 bp) were cloned and sequenced as described above.

2.5. Sequence analysis

Sequence analysis was performed using Sequencher (Gene Codes, Ann Arbor, MI, USA) and DNA Strider (Marck, 1992). Alignments and similarity matrices were calculated using ClustalW (Thompson et al., 1994). SeqVu (Garvan, 1996) was used to visualise sequence identities and hydrophobicities. The on-line servers SignalP (http://www.cbs.dtu.dk/services/SignalP/), PSORT (http://PSORT.nibb.ac.jp/) and Compute pI (http://expasy.hcuge.ch/cgi-bin/pi_tool) were used to predict N-terminal signal sequences, potential glycosylation sites and pI, respectively.

2.6. Southern analysis

Total genomic DNA was prepared from winter flounder (*Pleuronectes americanus* Walbaum), yellowtail flounder (*Pleuronectes ferruginea* L.) and Atlantic halibut (*Hippoglossus hippoglossus* L.), smelt (*Osmerus mordax* Mitchill), pollock (*Pollachius virens* L.), Atlantic salmon (*Salmo salar* L.) and rainbow trout (*Onchorynchus mykiss* Walbaum) and 7.5 μg digested with *Bam*HI and *Sst*I. Southern hybridisation was performed as previously described (Douglas and Gallant, 1998) using a gel-purified insert of the full-length amylase cDNA clone as a probe.

2.7. Reverse transcription-polymerase chain reaction (RT-PCR)

The presence of amylase mRNA was investigated using an RT-PCR assay. From the genomic and cDNA sequences of amylase, primers were designed that spanned intron-exon boundaries, had a T_m of over 50°C and little likelihood of secondary structure (see Table 2). Amplification of actin mRNA was performed to confirm the steady-state level of expression of a housekeeping gene and thus provide an internal control for amylase

Table 2
Oligonucleotide primers used in RT-PCR reactions
Forward slashes indicate position of intron/exon boundary.

Primer	Amino acid sequence	Nucleotide sequence (5′ > 3′)
RTAmy2	TR/NWGE	CACCAG/GAACTGGGGAG
RTAmy3	VNGK/DQ complement	CTGATC/CTTTCCGTTCAC

expression (Douglas et al., 1999a,b). Polyadenylated RNA was isolated from winter flounder adult intestine (20–50 mg), 10 whole larvae at 5, 13, and 20 days post-hatch (dph), 5 whole larvae at 27 dph, and 2 whole larvae at 41 dph as previously described (Douglas et al., 1999b) and one twentieth of the purified mRNA was reverse-transcribed using a Retroscript kit (Ambion, Austin, TX, USA). Aliquots of the reaction products were subjected to PCR with rTaq polymerase (Pharmacia, Uppsala, Sweden) using 1 μl of input cDNA. PCR conditions were: 1 min at 94°C; 32 cycles of 30 s at 94°C, 30 s at 52°C, 90 s at 72°C; 2 min at 72°C. Amplification products were resolved on a 2% NuSieve agarose gel using the 100 bp ladder for markers (Pharmacia) and the amount of each product was quantified using a GelDoc 1000 video gel documentation system (BioRad) with the Multianalyst software. Background signal was subtracted and the amount of amylase transcript calculated relative to the amount of actin transcript. Controls were performed using single primers (to eliminate single primer artifacts) and without reverse transcriptase (to eliminate amplification products arising from contaminating genomic DNA).

3. Results

Amplification of amylase proved to be extremely difficult. Annealing temperatures of 45°C and 35°C routinely used in PCR yielded no products and even at 30°C very faint signals were obtained. Nested PCR using amy1/3 followed by amy2/3 (Figs. 1 and 2), however, gave a 550-bp product. PCR products were cloned and several isolates were sequenced and shown to be amylase. The PCR product was used as a probe to isolate a full-length cDNA clone with an insert of 1.65 kb. Sequence analysis revealed an open reading frame of 512 amino acids followed by a polyadenylation site downstream of the stop codon and a poly A tail (Fig. 2).

PCR of winter flounder genomic DNA using primers amy2 and amy3 gave a 1.35 kb product. This product was cloned and several isolates were sequenced and shown to be very similar to the cDNA clone. The differences result in 16 changes in the deduced amino acid sequence; of these 10 are conservative and would be unlikely to affect

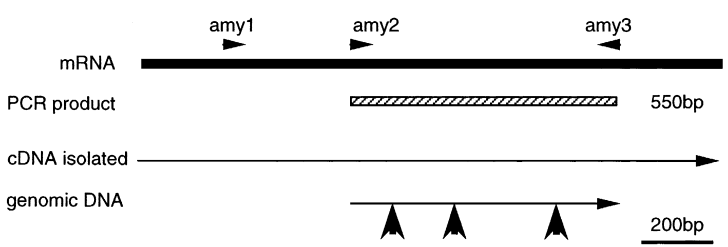
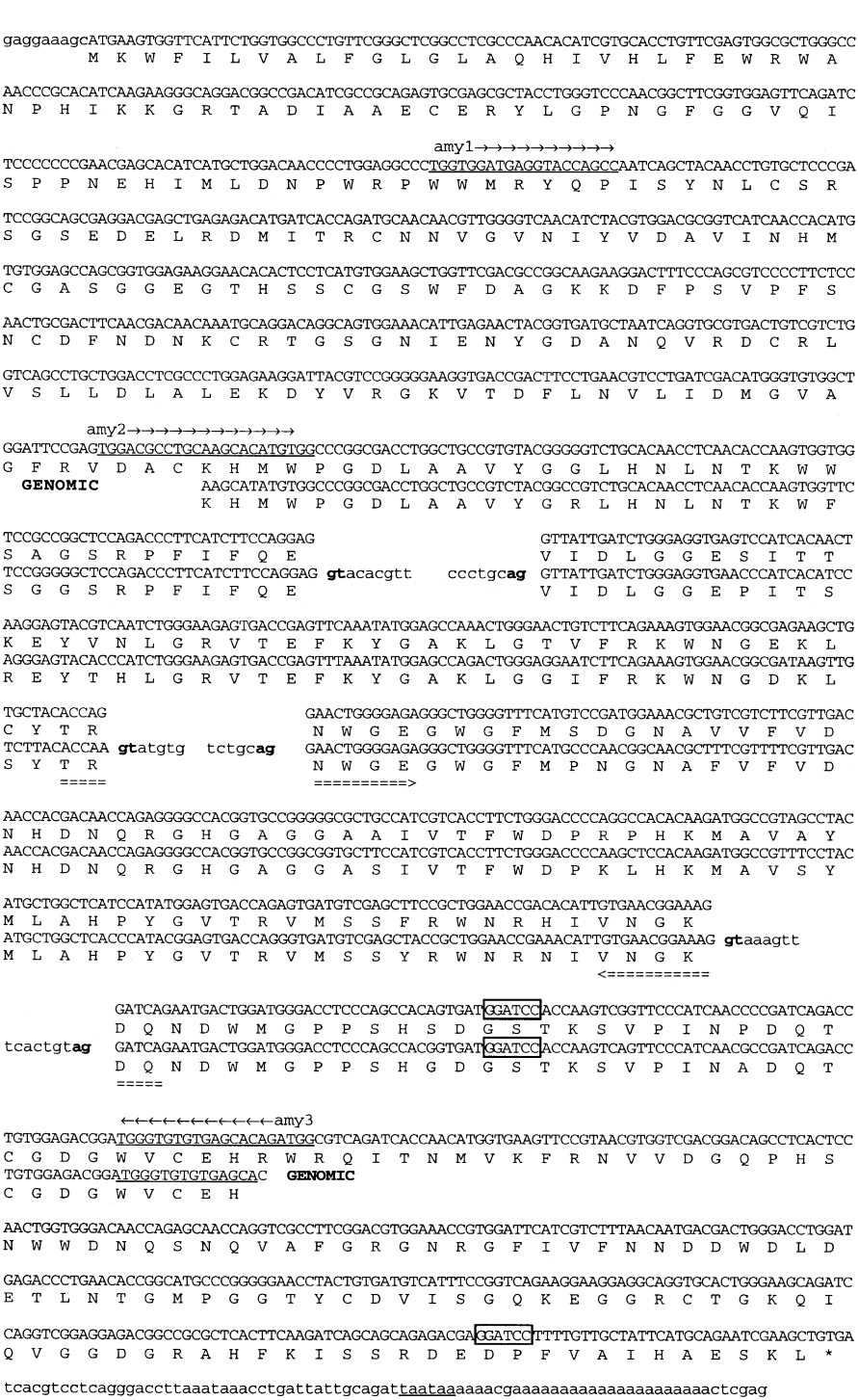


Fig. 1. Schematic of winter flounder amylase transcript. Arrowheads show positions of primers amy1, amy2 and amy3 and the hatched bar shows the position of the nested PCR product. Lines with arrows indicate lengths of amylase cDNA and genomic DNA isolated in this study. Introns in the genomic portion are indicated by large arrowheads.


amy1
amy2
amy3
GENOMIC
GENOMIC

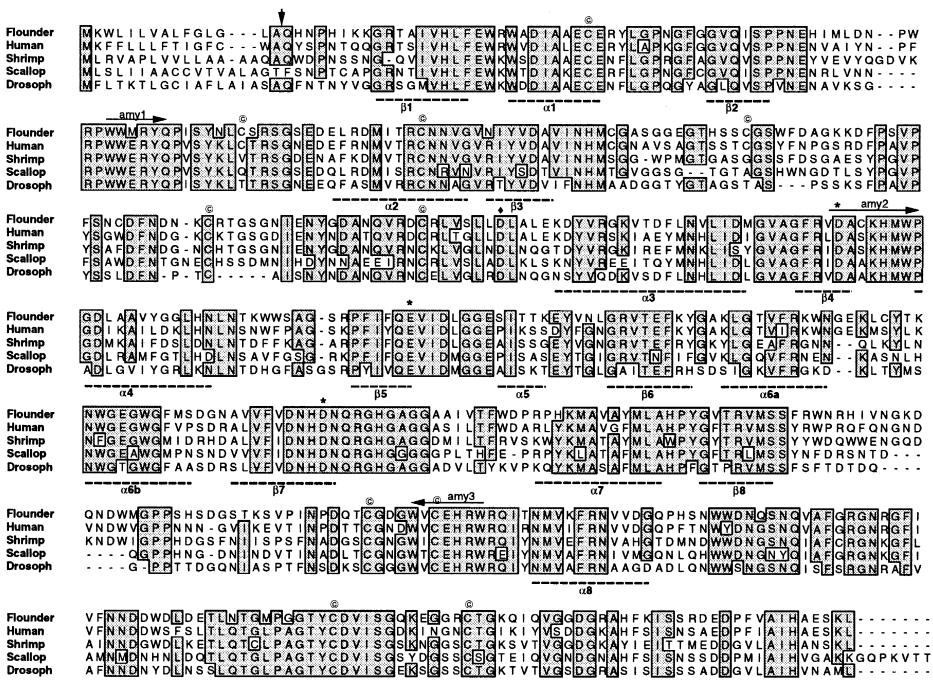


Fig. 3. Alignment of amino acid sequences of amylases from winter flounder, human 2A, shrimp, scallop and *Drosophila*. The predicted site of signal peptide cleavage is indicated by the arrowhead. The positions of primers amy1, amy2 and amy3 used for PCR experiments are indicated by arrows. Residues conserved in 80% of the sequences are shaded. Deletions are indicated by dashes and active site residues by asterisks. The aspartic acid residue involved in calcium binding is indicated by (♦) and the 10 cysteines involved in disulphide bridges in mammals are indicated by (©). Positions of α-helices and β-sheets are marked by dashed underlining.

secondary structure of the polypeptide or enzyme activity. Comparison of the cDNA and genomic sequences allowed the positions of three introns to be determined (Fig. 2), all of which possessed canonical GT/AG borders (Breathnach and Chambon, 1981).

Alignment of the deduced polypeptide sequence of the winter flounder amylase cDNA with homologs from human, shrimp, scallop and *Drosophila* is shown in Fig. 3. A potential cleavage site for a signal peptide was predicted to occur after amino acid 15. With the exception of the amino terminus, the rest of the molecule is highly conserved

Fig. 2. Alignment of cDNA and partial genomic amylase sequences of winter flounder. Coding sequence is in upper case and intron and non-coding sequences are in lower case. The full-length cDNA sequence and deduced polypeptide sequences are shown on top and the PCR amplified genomic sequence between primers amy2 and amy3 (underlined and indicated by arrows). The positions of primers RTAmy2 and RTAmy3 used for RT-PCR experiments are indicated by double-underlining. The GT/AG intron/exon boundaries are shown in bold face and the sizes of the introns in basepairs are indicated in brackets. A putative polyA addition site is underlined and the two *Bam*HI sites are boxed.

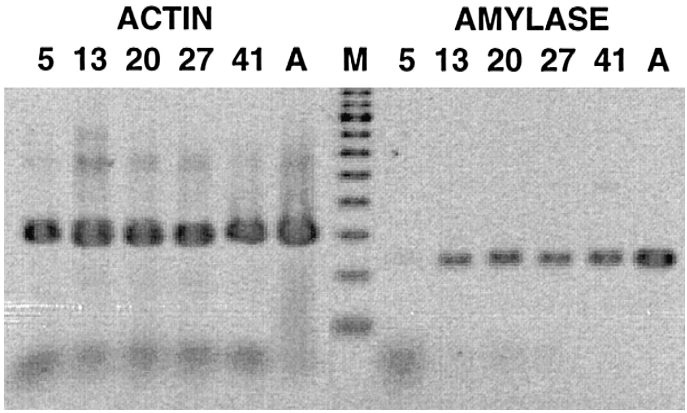


Fig. 4. RT-PCR assay of amylase expression in developing larvae. Samples were larvae at 5 dph (lane 1), 13 dph (lane 2), 20 dph (lane 3), 27 dph (lane 4), 41 dph (lane 5) and adult (lane 6). Amplification products from reactions using primers specific for actin (310 bp) and amylase (235 bp) were resolved on a 2% agarose gel using a 100 bp ladder (Pharmacia) as markers (lane M).

among these five organisms. The winter flounder sequence shows the highest similarity to that of human (70%) followed by shrimp (60%), scallop (56%) and *Drosophila* (53%).

Assays of amylase gene expression based on RT-PCR showed that, relative to the housekeeping gene actin, amylase transcripts were present in slight amounts as early as 5 dph, and steadily increased between 13 and 20 dph (Fig. 4; Table 3). At metamorphosis (27 dph), the level of expression decreased slightly, but in adult was at high levels. The amount of actin product varied by only 5% during the duration of the experiment showing that it provides a good internal control.

Southern hybridisation analysis showed that the winter flounder amylase probe was easily able to detect homologs in other flatfish (halibut and yellowtail flounder), but only gave faint signals with pollock and smelt DNA and no signal with salmon or rainbow trout DNA. Two large *Sst*I fragments and four smaller *Bam*HI fragments hybridise to winter flounder DNA, consistent with sequence data indicating two copies of the amylase gene. No *Sst*I sites are present in either cDNA or the partial genomic clone and two *Bam*HI sites are present in both. Although no information on restriction sites in the

Table 3
Amount of amylase transcript relative to actin transcript

Age (dph)	Amylase transcript
5	0.06
13	0.96
20	0.99
27	0.75
41	0.87
Adult	1.68

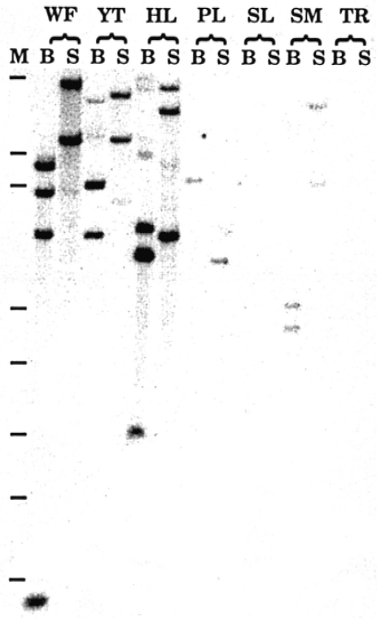


Fig. 5. Southern hybridisation analysis of winter flounder (WF), yellowtail flounder (YT), halibut (HL), pollock (PL), salmon (SL) smelt (SM) and trout (TR) amylase genes using the restriction enzymes *Bam*HI (B) and *Sst*I (S) and the winter flounder amylase probe. Markers (M) are lambda DNA digested with *Sty*I (24.0, 7.7, 6.2, 3.4, 2.7, 1.9, 1.4, 0.9 kb).

remainder of the amylase gene is available at this time, the absence of additional *Sst*I sites and the presence of one additional *Bam*HI site in an unsequenced intron would give the pattern seen in Fig. 5.

4. Discussion

4.1. Genes

Amylase genes have been isolated from a variety of organisms including bacteria, fungi, plants and animals. Mammalian α-amylases are of two main types: salivary *amy*1 (Nishide et al., 1986) and pancreatic *amy*2 (Horii et al., 1987; Gumucio et al., 1988; Yokouchi et al., 1990). These genes are expressed in a tissue-specific manner. Seven α-amylase genes are clustered in the human genome — two pancreatic amylase genes, three salivary amylase genes, and two truncated pseudogenes (Gumucio et al., 1988). Since fish do not possess salivary glands, they only produce pancreatic α-amylase and it is likely that the two sequences isolated from winter flounder are analogous to *amy*2A and *amy*2B of human. Mouse is reported to contain four genes for pancreatic amylase and one for salivary amylase (Hjorth et al., 1980). Two families of α-amylase including at least five genes have been detected in shrimp (van Wormhoudt and Sellos, 1996).

Insects (Levy et al., 1985) and rats (MacDonald et al., 1980) also contain high numbers of these genes.

Among animals, cDNAs for amylase have been isolated from rat (Rutter, 1980), pig (unpublished, GenBank accession AF064742), mouse (Hagenbuchle et al., 1980), human (Nakamura et al., 1984), several insects (Boer and Hickey, 1986; Grossman and James, 1993), shrimp (van Wormhoudt and Sellos, 1996) and, recently, scallop (Le Moine et al., 1997). Until this study, none have been reported from any fish. As shown in Fig. 3, the winter flounder amylase most closely resembles the human amylase both in terms of percent identity (70%), shared deletions and presence of the 10 conserved cysteines that participate in disulphide bond formation.

By comparing the genomic and cDNA sequences, three introns were detected in the amplified portion of the winter flounder amylase gene, the positions and phases of which are identical to those found in human. However, in human amylase genes there is an additional intron between introns 2 and 3 of winter flounder. The pancreatic α-amylase gene of mouse and man is interrupted by nine introns (Horii et al., 1987) whereas that from salivary gland contains one extra intron near the 5′ end of the gene (Nishide et al., 1986). Amylase genes in *D. melanogaster* have no introns (Boer and Hickey, 1986) and those of *D. pseudoobscura* and many other non-drosophilid insects harbour one short intron (Da Lage et al., 1996).

4.2. Predicted polypeptide

The predicted amylase polypeptide is 512 amino acids, including a putative 15 amino acid signal peptide, and it is similar in size to other sequenced amylases. The first amino acid after the signal cleavage site is a glutamine, which corresponds to the blocked end of the mature protein in shrimp (van Wormhoudt and Sellos, 1996). The positions of the three acidic residues that form the catalytic centre and the aspartic acid that is in the calcium-binding centre (Pasero et al., 1986; Janacek, 1994) are all preserved. The molecular weight of the mature protein is 55,769 Da, similar to that of other animals. The predicted isoelectric point is 6.76, similar to that of scallop, human and *Drosophila* but one unit higher than that of shrimp. There is one potential N-glycosylation site (N-X-S/T) at position 428–430.

Mammalian amylases are characterised by an eight-barrel (α/β) secondary structure and the winter flounder sequence aligns well with the human sequence in these conserved regions suggesting that it also contains eight α-helical and β-sheet structures (Fig. 3). Interestingly, all 10 of the cysteines involved in disulphide bond formation in mammals (Janacek, 1994), but not molluscs, crustaceans or insects, are preserved in the winter flounder.

4.3. Gene expression

The RT-PCR assay developed in this study allowed an accurate and sensitive estimation of amylase gene expression from larval fish at very early stages of development (as early as 5–13 dph). By designing primers that span intron/exon borders in addition to DNase digestion of RNA samples, the possibility of obtaining signal from trace amounts of DNA in the RNA preparations was reduced. Also, the control

experiments with single primers removed the possibility of mistakenly scoring single-primer artifacts. Comparison of amylase transcript band intensities using video densitometry with those of the housekeeping gene, actin, allowed the relative amount of amylase transcript to be quantified.

In teleost fish as exemplified by mosquitofish, there is a progressive increase in amylase expression during development (Yardley, 1988). Sole (Boulhic and Gabaudan, 1992), turbot (Segner et al. 1994) and summer flounder (Bisbal and Bengtson, 1995) contain zymogen granules in the pancreatic cells by first-feeding (3–5 dph) and amylase activity was detected at this early stage in sole (Ribeiro et al., 1999) and turbot (Cousin et al., 1987). The pancreas of Japanese flounder contains zymogen granules at 3 dph and exhibited a positive reaction with antibodies to another pancreatic enzyme, trypsinogen (Kurokawa and Suzuki, 1996). Digestive enzymes were also detected immunohistologically in the digestive tract of larval winter flounder at 3 dph (Baglole et al., 1998).

Winter flounder larvae are herbivorous, consuming algae in the early stages of development and then rotifers. Both of these food sources require amylase for digestion, and mRNA levels in our study increased through to metamorphosis (Fig. 4; Table 3). Amylase mRNA levels decreased at metamorphosis (after 20 dph) in winter flounder, in agreement with studies on amylase enzyme activity in developing Japanese flounder (Tanaka et al., 1996). A decrease in amylase specific activity during development was also reported in sea bass fed live prey (Cahu and Zambonino Infante, 1994) and a decrease in amylase mRNA was reported between 20 and 29 dph in sea bass (Peres et al., 1998).

Amylase activity has been reported to be higher in omnivorous fish than in carnivorous fish (Hidalgo et al., 1999). Amylase expression is repressed by glucose and induced by starch in *Drosophila* (Benkel and Hickey, 1986, 1987; Inomata et al., 1995). However, no glucose repression or starch induction has been observed in the housefly, *Musca* (McCommas and Shornick, 1990), indicating that this is a species-specific phenomenon. In shrimp, *Penaeus vannamei*, increasing dietary casein or the ratio of casein to starch caused the disappearance of expression of one of two amylase isoforms (Le Moullac et al., 1996). In larval sea bass, amylase specific activity was increased by increasing dietary starch content but mRNA levels were unaffected (Peres et al., 1998).

Little is known of the protein requirements of larval winter flounder and published studies utilised artificial feeds formulated for other fish species (Lee and Litvak, 1996). These are generally high-protein diets, containing at least 50% crude protein. Given the relatively high amounts of amylase present in 20 dph winter flounder, and the suggestion that early weaning would be beneficial, the replacement of some fraction of protein by carbohydrate may result in a cheaper alternative feed. Using the RT-PCR assay developed here, it should be possible to assess the level of amylase gene expression resulting from different diets and to look for specific ingredients that can induce amylase expression in flatfish.

Preliminary studies have shown that the primers used in this assay can also be used with halibut larvae (Douglas, Gallant and Gawlicka, unpublished). Furthermore, amylase sequences were successfully amplified from genomic DNA of the black cod, *Anaplopoma fimbria* Pallas, 1814 (data not shown), indicating that this assay may be applied to more distantly related fish.

Acknowledgements

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