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IMPROVING GENETIC STOCKS OF ATLANTIC SALMON BY USING GENE(S) CODING FOR TRYPSIN-LIKE ISOZYMES

(Sluttrapport NFR/NFFR- nr. 1402 - 701.300)

Forbedring av avlsstammer for Atlantisk laks ved bruk av gener som koder for trypsin-like isozymer

HAVFORSKNINSTITUTTET
SENTER FOR HAVBRUK

Trypsin isozym *TRP-2(92)*, en markør for vekst i Atlantisk laks.

Trypsin er et av de viktigste fordøyelsesenzymene i tarmen og finnes i flere former, isozymer, som kan ha forskjellige egenskaper. Tilstedeværelse av ett av disse isozymene er assosiert til økt vekst i laks og røye. Undersøkelser av genene for trypsin i laks viser minst 8 forskjellige. Detaljstudier av disse ved bestemmelse av deres aminosyre sekvens kan fortelle noe om forskjellen mellom variantene av trypsin på molekylnivå. De gir også muligheter til utvikling av enklere metoder for utvelgelse av laks med isozym assosiert til forbedret vekst.

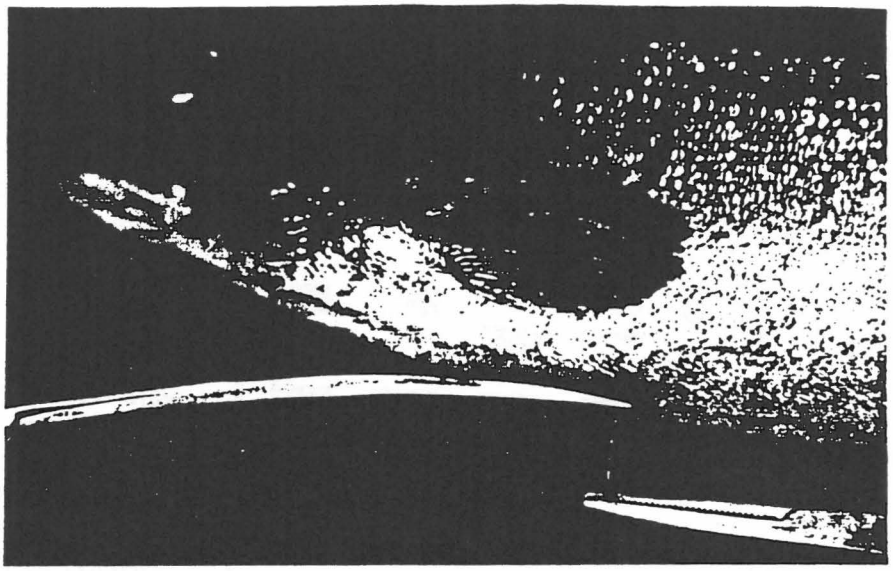


Fig. 1. Biopsi fra blindtarm fra laks.
For å kunne undersøke fordøyelsesenzymer må det taes en prøve av tarmvevet hos fisken. Vevsprøven taes fra bedøvde fisk ved å trekke litt av tarmen ut gjenneom et snitt i buken og klippe av en liten bit. Etter prøvetaking lukkes snittet med en heftemaskin. Overlevelsen er meget god (95 %).

Isozymer av trypsin fra blindtarm i laks kan studeres ved isoelektrisk fokusering i agarose gel (Fig. 2). Vi har vist at tilstedeværelse av isozymet *TRP-2(92)* er assosiert med økt vekst. Sammenhengen er synlig gjennom hele livssyklus til fisken både før og etter smoltstadiet foruten at den er tilstede både på individ og familie nivå. Tilstedeværelse av isozymet syne ikke å ha effekt på kvaliteten av egg, men en mulig bedret klekke suksess er observert. Studier av tre populasjoner av laks indikerer at høyere frekvens av dette isozymet gir høyere vekst rate av laksestammene (se tabell).

Arktisk røye.

Dette fenomenet er også observert i arktisk røye. En anadrom raskt voksende Hammerfest stamme har et trypsin isozym som likner *TRP-2(92)* allelet i Atlantisk laks. Dette isozymet ikke finnes i en ikke-anadrom sakte voksende Skogseid stamme som lever ved noe høyere vanntemperatur sør i Norge. Effekten av tilstedeværelse av isozymet synes å manifesteres ved lav vanntemperatur i begge arter.

Trypsin, ett fordøyelsesenzym.

Trypsin bryter ned kost proteiner til korte peptider. Andre fordøyelsesenzymer som bryter ned disse peptidene til opptagbare næringsstoffer til bruk i kroppens proteinsyntese. Siden laks krever et høyt innhold av kost proteiner for god vekst regnes trypsin som en primær faktor for kroppens protein syntese. Trypsin har også en annen viktig rolle ved at det aktiverer to andre fordøyelsesenzymer i tarmen, chymotrypsin og elastase. Vi har observert assosiasjon mellom trypsin isozymet *TRP-2(92)* og absorpsjon (i laks) og utnyttelse (begge arter) av kost proteiner. Det er derfor mulig at tilstedeværelse av *TRP-2(92)* allelet ikke bare er en genetisk markør, men har også direkte innflytelse på fiskens opptak og utnyttelse av proteiner i foret.

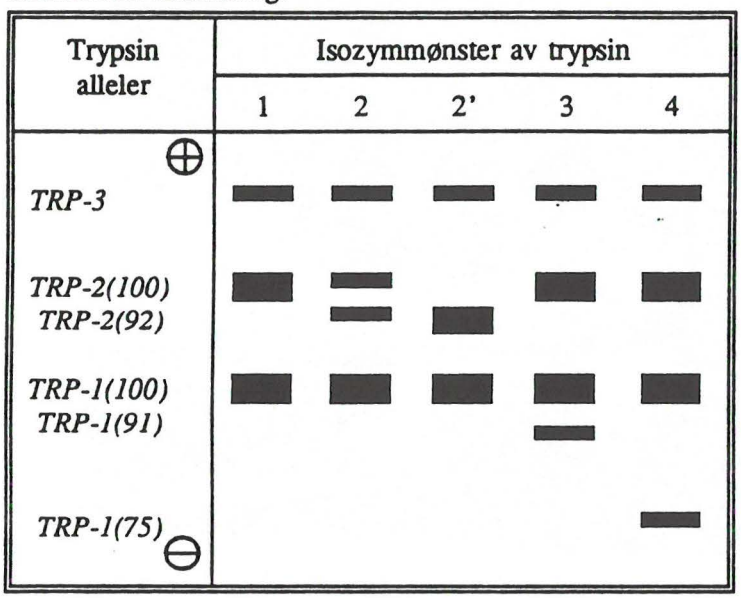
Trypsin gener, ett nytt hjelpemiddel i selektiv avl.

Bestemmelse av isozymmønsteret av trypsin krever et inngrep i fisken for å få tak i en del av tarmen som utgangsmateriale for undersøkelsen (Fig. 1).

Table 1. Gjennomsnittlige vekter av laks med og uten trypsin isozym *TRP-2(92)* fra tre forskjellige elve-stammer. (f=Frekvens av isozym *TRP-2(92)*. Gjennomsnitts vekter i samme periode merket med forskjellige subskrifter (a og b), eller med *, er signifikant forskjellige (P<0.04).

Lakse stammer	Vekt±SEM (g), Oktober 90		Vekt±SEM (g), Februar 91	
	Med	Uten	Med	Uten
Dale (f=0.29)	$52.7\pm2.2_a$	$42.6\pm1.2_b$	$64.3\pm2.9_a$	$50.2\pm1.5_b$
Lonevåg (f=0.10)	$47.9\pm2.8_a$	$40.7\pm0.8_b$	$*55.0\pm4.0$	$*47.5\pm1.0_b$
Voss (f=0.09)	$40.3\pm1.7_b$	$40.5\pm0.7_b$	$51.2\pm2.0_b$	$49.0\pm0.7_b$

Fig. 2. Skjematisk presentasjon av trypsin isozymer etter isoelektrisk fokusering.



Ofte ønsker en å holde fisken i live etter undersøkelsen for nye tester eller evt. avl. Ved å studere arvestoffet, DNA, kan vi i teorien oppnå de samme resultater med utgangspunkt i hvilket som helst vev fra fisken, f.eks en del av en finne. En slik metode ville representere en betydelig forenkling av arbeidet. For å utarbeide en slik målemetode basert på DNA er trypsin klonet fra pancreatisk vev fra laks. Ved denne undersøkelsen er så langt fem varianter av trypsin identifisert. Alle er fullstendig karakterisert med hensyn på aminosyre rekkefølge. Generelt viser alle stor grad av identitet ved sammenligning med kjente trypsin fra mammalia, omlag 69 % identitet. Alle essensielle deler av proteinet er konserverte så som aminosyrer i enzymets aktive sete, residuer som gir interne kryssbroer etc. Trypsin synes derfor å være et relativt sterkt konservert gjennom evolusjonen. Imidlertid skiller en av variantene i laks seg fra de fire andre like mye som trypsin fra laks skiller seg fra rotte. Ved nærmere analyse synes den ene varianten å representere gammelt enzym i evolusjons sammenheng og representerer en cationisk form av enzymet. De fire andre synes å representere anioniske former. Denne cationiske formen synes fra våre resultater å være tilstede hos alle individer. Variasjonen i trypsin mønster synes å ligge i de anioniske formene.

Mange gener for trypsin i laks.

Analyser av DNA fra laks viser et komplekst mønster av trypsin gener minst 8 i alt. Arbeidet videre vil konsentreres om å sammenlikne genmønsteret for trypsin fra forskjellige grupper av laks for å utvikle en DNA basert metode som kan erstattet tradisjonelle isozymmønster bestemmelser. Det vil også bli forsøkt å identifisere de forskjellige kloner av trypsin i forhold til isozymmønsteret. Kunnskap fra dette prosjektet kan også trolig anvendes på andre fiskearter da trypsin er sterkt konservert gjennom dyrerekken.

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Improving genetic stocks of Atlantic salmon by using gene(s) coding for trypsin-like isozymes

(Forbedring av avlsstammer for Atlantisk laks ved bruk av gener som koder for trypsin-like isozymer)

INTRODUCTION

Previous studies of Atlantic salmon, *Salmo salar* L., (Gunnes & Gjedrem, 1978), rainbow trout, *Oncorhynchus mykiss* Walbaum, (Gall & Gross, 1978; Ayles & Baker, 1983), brook trout, *Salvelinus fontinalis* Mitchill, (Cooper, 1961) and Arctic charr, *Salvelinus alpinus* L., (Holm, 1989; Berg, 1989; Barnung & Holm, 1991), have shown significant differences in growth rate between strains. Genetic variation in metabolic enzymes has been studied and some isozymes could differentiate and clarify the genetic structure of different populations of rainbow trout (Utter & Hodgins, 1972; Guyomard, 1984), brown trout, *Salmo trutta* L., (Taggart *et al.*, 1981; Guyomard & Krieg, 1983) and Atlantic salmon (Cross & Payne, 1977; Cross & Ward, 1980; Ståhl, 1981). Differences in growth were found in rainbow trout in association with genetic differences in phosphoglucomutase (Allendorf *et al.* 1983) and in transferrin (Reinitz 1977). An association between genetic variation in trypsin-like isozymes and fish size was found in a mixed population of Atlantic salmon fry (Torrissen 1987), and in the growth rate of Atlantic salmon from smolt until maturation (Torrissen, 1991). This difference was associated with the presence or absence of the trypsin-like isozyme variant *TRP-2(92)*. Torrissen & Barnung (1991) also found that a fast growing "Hammerfest" strain of Arctic charr possessed a similar *TRP-2(92)* allele, while this allele did not exist in the Arctic charr of the slow growing "Skogseid" strain. The effect of this isozyme allele seemed to be manifested at low temperature, both in Atlantic salmon (Torrissen, 1991) and Arctic charr (Torrissen & Barnung, 1991). The advantage of using trypsin isozyme patterns as a biological genetic marker suitable as an indirect trait for selective breeding has been observed. Since trypsin-like isozymes are digestive proteases, differences in growth between the fish with and without the *TRP-2(92)* allele could possibly be linked directly to variation in trypsin activity. From our hypotheses, this could be caused by several factors:

a. differences in protein digestibility,
b. differences in the dissociation constant (K_m) of the enzyme-substrate complex, especially at low temperature, which affects
c. differences in digestion rate, leading to
d. differences in feed consumption.

Variation in catalytic efficiency (k_{cat}/K_m) was observed among three trypsins from the pyloric caeca of Atlantic cod (Ásgeirsson *et al.* 1989). Besides from mammalia, complete protein sequence of trypsin was only studied in dogfish (Titani *et al.* 1975), and N-terminal analysis of trypsin was carried out such as, in Atlantic salmon (Sletten 1988, cited from Smalås 1990), lungfish (Hermodson *et al.* 1971), and Atlantic cod (Ásgeirsson *et al.* 1989). Trypsin from fish has never been cloned before. Study of molecular differences among trypsin isozymes

from Atlantic salmon may lead to some understanding of the relationship between the basic structure of proteins and the enzymatic activity. By this way, the gene(s) coding for the enzymes have to be identified and cloned. Furthermore, study of trypsin gene(s) can lead to a development of an alternative method for screening individual fish, DNA polymorphism technique, which could be able to differentiate the salmon simply by their fins, which are easier sampled than biopsy of pyloric caeca (Fig. 1). This technique will be useful for improving genetic stocks of Atlantic salmon for future breeding programme.

This project is a collaborative work between Matre Aquaculture Research Station, Institute of Marine Research, and Center of Biotechnology, University of Bergen.

The objectives of this project are divided into two main aspects:

1. Biochemical and nutritional aspects

a. To study heredity of trypsin isozymes.
b. To study the effect of trypsin isozyme *TRP-2(92)* on growth of three different salmon populations.
c. To study whether the trypsin isozyme *TRP-2(92)* is associated with growth through differences in digestion and utilization of dietary protein.

Personels involved are:

Krisna R. Torrissen, senior scientist at Matre Aquaculture Research Station.
Torunn Opdal, laboratory assistance at Matre Aquaculture Research Station.

2. Biotechnological aspect

a. To identify and clone trypsin(s) from the pancreas of Atlantic salmon.
b. To develop a method based on DNA polymorphism to differentiate salmon with different trypsin isozymes.

Personels involved are:

Rune Male, associate professor at Center of Biotechnology.
Marit Flo Jensen, biological engineer at Center of Biotechnology.

Publications

Torrissen K.R. & Shearer K.D. (1992) Protein digestion, growth and food conversion in Atlantic salmon and Arctic charr with different trypsin-like isozyme patterns. *Journal of Fish Biology* **41,** 409-415.

Male R., Lorens J.B., Jensen M.F. &**Torrissen K.R.** (1992) Cloning and sequencing of two trypsin cDNA's from Atlantic salmon (*Salmo salar*) *The International Symposium on Cultivation of Atlantic salmon*, 16-20 August 1992, Bergen, Norway. Abstract No. 36.

Torrissen K.R., Lied E. & Espe M. (1992) Genetic differences in postprandial plasma and muscle free amino acids in the Atlantic salmon with different trypsins. *The International Symposium on Cultivation of Atlantic salmon*, 16-20 August 1992, Bergen, Norway. Abstract No. 37.

Torrissen K.R., Male R. & Nævdal G. (1993) Trypsin isozymes in Atlantic salmon (*Salmo salar* L.): Studies of heredity, egg quality and effect on growth of three different populations. *Aquaculture and Fisheries Management* **24**, 281-289.

Male R., Lorens J.B., Smalås A.O., Jensen M.F. & **Torrissen K.R.** Cloning and characterization of cationic and anionic forms of trypsin from Atlantic salmon. (in prep.)

BIOCHEMICAL AND NUTRITIONAL ASPECTS

A. Heredity of trypsin isozymes

Abstract

Isoelectric focusing of Atlantic salmon (*Salmo salar* L.) trypsin resolved three major isozymes designated *TRP-1*, *TRP-2* and *TRP-3*, with three variants, *TRP-1(91)*, *TRP-1(75)* and *TRP-2(92)*. The *TRP-1* and *TRP-2* may represent duplicated loci which exhibit either tetrasomic or disomic inheritance from pairing of the homeologous chromosomes.

Materials and methods

Smolts from twenty-six families of known parental trypsin genotypes were collected and the pyloric caeca from about 50 fish from each family were randomly biopsied and analysed for trypsin isozyme patterns by isoelectric focusing on agarose gel according to Torrissen (1991).

Results and discussion

The major trypsin isozyme patterns and the designated alleles are illustrated in Fig. 2. Five major isozyme patterns and their combinations were observed as described by Torrissen (1987). Two polymorphic loci, the *TRP-1* with variants *TRP-1(91)* and *TRP-1(75)*, and the *TRP-2* with a variant *TRP-2(92)*, were detected.

The observed phenotypic distributions of the *TRP-1* among the different progenies and the estimated *TRP-1* genotypes of the parents are shown in Table 1. The distribution of the *TRP-2* and the observed isozyme phenotypes of the parents are shown in Table 2. In order to fit the parental phenotypes with the observed phenotypic frequency distribution in the offspring, the estimated parental genotypes of both polymorphic loci, *TRP-1* (Table 1) and *TRP-2* (Table 2), were assumed to be duplicated loci, with either disomic or tetrasomic inheritance. An incidence of duplicated locus *TRP-2*, with *TRP-2(100)* and *TRP-2(92)* alleles, was observed (Torrissen 1987). Discrepancy between observed and expected isozyme patterns in the offspring compared to patterns in the parents were observed. Undetected isozyme

expression is apparent, for example in female b of families 3 and 11 and in female c of families 4 and 6 (Tables 1 and 2). The presence of the *TRP-1(91)* gene in these females was inferred, because of the presence of this phenotype in the offspring (Table 1). Similarly, the presence of the *TRP-2(92)* gene was indicated by its observation in the offspring of family 4 and homozygote phenotype observed in families 6 and 11 (Table 2). Undetected expression of *TRP-2(100)* was deduced in male m of families 11 and 20, and in other males from families 7, 14, 23 and 24 (Table 2). Studies of half-sib families prevented erroneous verification of the isozymes at low frequency. The presence of isozyme *TRP-1(75)* in family 3 and *TRP-1(91)* in family 18 (Table 1) may be erroneous, because these phenotypes were not observed in the parents. Undetected isozyme expression may be attributed to either gene suppression or isozyme expression below the level of detection.

Crossing of individual fish resulted in offspring with trypsin isozyme patterns which cannot be easily explained by ordinary disomic (Mendelian) inheritance. This phenomenon has been observed for several salmonid enzymes (Allendorf & Thorgaard 1984; Hartley 1987), and is postulated to be the consequence of a tetraploid event in an ancestral salmonid. Subsequent genome rearrangements (e.g. Robertsonian translocation) resulting in reduced chromosome number, have reestablished disomic inheritance at approximately 75% of salmonid loci (Allendorf & Thorgaard 1984). In the modern salmon, it has been shown that some gene loci still demonstrate tetrasomic inheritance, furthermore, the generation of non-parental types, possibly the result of recombination between homeologous chromosome pairs at meiosis, produces offspring approaching tetrasomic expectations (Allendorf & Thorgaard 1984; Wright *et al.* 1983). The results reported in this paper do not easily fit either explanation. This could be attributed to the experimental method employed, which may underestimate the number of heterozygotes if the isozyme alleles exist as a 3:1 ratio.

B. Effect of trypsin isozyme *TRP-2(92)* on growth of three salmon populations

Abstract

Studies of three salmon populations suggested a correlation between the presence of higher frequency of trypsin isozyme *TRP-2(92)* and better growth rate.

Materials and methods

Three different strains, Dale, Lonevåg and Voss, of Atlantic salmon parr from the western part of Norway were studied for the frequency of trypsin isozyme *TRP-2(92)* and their growth over a four month period in the winter of 1990/1991. Pyloric caecae biopsies were taken from about 150 fish from each strain, their trypsin isozyme patterns analysed and the fish individually marked by "Floy" anchor tags. The fish were cultured at Selstø Sea Ranching Station, Sotra. They were weighed at the start and end of the experiment. The specific growth rate of each strain was calculated using the method of Houde and Schekter (1981):

where, $$\text{SGR (\% per day)} = 100(e^{g} - 1)$$

and, $g = (\ln W_t - \ln W_0) / (t - t_0)$, W_t = weight at day t, W_0 = weight at day t_0

Results and discussion

The trypsin isozyme *TRP-2(92)* frequency in Atlantic salmon parr from the three different strains was investigated. The Dale strain (45.7 ± 1.1 g), which had higher frequency of the isozyme (f=0.29), was significantly larger (P<0.01) than the Lonevåg (42.1 ± 0.7 g) and Voss (40.4 ± 0.6 g) strains which displayed the frequencies of 0.10 and 0.09, respectively. After four months at low water temperature (2-3°C), the daily growth rates were similar between the Dale and the Voss strains (0.15 ± 0.01 %/day), but significantly higher (P<0.02) than that of the Lonevåg strain (0.12 ± 0.01 %/day). The *TRP-2(92)* salmon from the Dale and the Lonevåg strains were significantly heavier (P<0.02) than those lacking this isozyme, but not in the Voss strain (Table 3). A growth difference between Voss strain with and without the isozyme *TRP-2(92)* may possibly be observed during the fast growing sea-phase, as this strain grows much faster in sea-phase than in freshwater-phase, relative to the other strains (M. Holm, personal communication). The *TRP-2(92)* salmon of Dale and Lonevåg strains were significantly larger (P<0.04) than that of Voss strain at the start of the experiment. Because of slower growth rate in Lonevåg strain, the average weights between the *TRP-2(92)* salmon of Lonevåg and Voss strains were not significantly different at the end of the experiment. No difference in weight was observed among the salmon lacking the isozyme from different strains. No trypsin allele *TRP-1(75)*, pattern 4 (see Fig. 2), was observed among these strains.

These data suggest a correlation between higher growth rate and high frequency of *TRP-2(92)* salmon.

C. Effect of trypsin isozyme TRP-2(92) on digestion and utilization of dietary protein

Abstract

Protein digestibility and food conversion were determined in groups of Atlantic salmon (*Salmo salar* L.) and Arctic charr (*Salvelinus alpinus* L.) with or without the trypsin-like isozyme *TRP-2(92)*. Determinations were made at two salinities, 0 and 27 ppt; at two temperatures, 6 and 10 °C and on large (200 g) and small (30-90 g) fish. The overall digestibility of protein was found to be 81.4±0.2 percent and was unaffected by the presence of the isozyme or the other variables. The feed conversion ratio of Atlantic salmon possessing *TRP-2(92)* was observed to be significantly lower with significantly higher specific growth rate than in fish without this isozyme.

Materials and methods

The fish were divided into 2 groups, with or without *TRP-2(92)* allele, using the methods of Torrissen (1987, 1991) for Atlantic salmon, and Torrissen and Barnung (1991) for Arctic charr.

Digestibility was determined by chromic oxide method. The fish were fed to excess by automatic feeders with 52 percent protein feed containing 0.3 percent chromic oxide (dry basis), re-pelleted from a commercial feed (Tess Elite Plus, T. Skretting A/S, Stavanger, Norway).

For feed utilization experiment, the fish were fed a fixed ration of 0.5 percent of their initial body weight per day. Individual fish were weighed approximately fortnightly to control the amount of feeding.

All fish in each experiment were labelled with "Floy" anchor tags. The Atlantic salmon were biopsied at least 2.5 months before the start of the experiments with less than 5% mortality. Individual fish weight was measured during the experimental periods.

The apparent digestibility coefficient (ADC) of protein was calculated according to the following formula:

$$ADC\ (\%) = 100(a - b)/a$$

where $a = \frac{\%\text{Protein in feed}}{\%Cr_2O_3 \text{ in feed}}$ $b = \frac{\%\text{Protein in faeces}}{\%Cr_2O_3 \text{ in faeces}}$

The specific growth rate (SGR) was calculated using the method of Houde and Schekter (1981):

where, $$SGR\ (\%\ \text{per day}) = 100(e^g - 1)$$

and, $g = (\ln W_t - \ln W_0) / (t - t_0)$, W_t = weight at day t, W_0 = weight at day t_0

Results

Digestibility experiments

No statistical difference in the ADC of protein was observed between the groups with and without trypsin isozyme *TRP-2(92)*, either in Atlantic salmon or Arctic charr.

The average ADC's of Atlantic salmon smolts of the groups with and without the variant isozyme *TRP-2(92)* were 81.8 and 82.7 percent, respectively (Table 4), and those of Atlantic salmon post-smolts were 81.1±0.3 percent and 81.5±0.3 percent, respectively (Table 5). Between the two strains of Arctic charr, the average ADC's were 82.9±1.2 and 80.6±0.2 percent for the fast growing "Hammerfest" strain and the slow growing "Skogseid" strain, respectively (Table 4).

Feed utilization experiments

Atlantic salmon smolts

During the 55 days, the fish were fed a restricted ration, the fish possessing the isozyme variant were apparently better able to utilize the feed since their growth rate (based on individuals) was significantly higher (P=0.02) (Table 6). About 7 percent difference in weight gain was observed during this experimental period (P=0.04). For the group with the variant, the feed conversion ratio (FCR) was significantly lower (P=0.02) with significantly higher (P=0.01) in protein efficiency ratio (PER) than that of the group without the variant.

Arctic charr

During 36 days with restricted ration, the fast growing "Hammerfest" strain appeared to utilize the feed better than the slow growing "Skogseid" strain (Table 6). The feed conversion ratios (FCR) were 0.66 and 0.82 for the fast growing and the slow growing strains, respectively. Statistical analysis could not be performed because there was not enough replication during this experiment. The specific growth rate (based on individuals) is significantly higher (P=0.02) in the fast growing (0.69±0.04) than in the slow growing (0.52±0.07) strains.

Discussion

During the digestibility experiment, the salmon smolts and the charr consumed a small amount of feed, perhaps due to decreasing water temperature. The temperature decreased to 7.5°C and 4.4°C in November and December, respectively. A reduction in feed intake due to declining temperature was also observed by Atherton & Aitken (1970). No differences in the ADC of protein were found in any case. These findings therefore disagree with those of Austreng & Refstie (1979) who reported differences in protein digestibility among families of rainbow trout. The overall average ADC of protein in Atlantic salmon smolts (Table 4), Atlantic salmon post-smolts (Table 5), small Arctic charr ("Skogseid" strain) and large Arctic charr ("Hammerfest" strain) (Table 4) was 81.4±0.2 percent. The variation in water temperature from 4.4°C to 11.1°C, in salinity from 0 ppt to 27 ppt, in fish size from 30 g to 200 g, and in salmonid species (Atlantic salmon and Arctic charr) had no apparent effect on the ADC of protein. The results support the statement of Hepher (1988), that digestibility in fish is regulated quite efficiently, and except for the effect of feed composition, digestibility is unaffected under varying conditions, such as fish size, temperature and salinity.

At temperatures below 6°C, the slow growing "Skogseid" strain did not consume as much feed as the fast growing "Hammerfest" strain, since very little faeces were obtained. At temperatures higher than 6°C, faeces were easily collected from both strains because feed consumption increased. These results showed that, at low temperature, feed consumption patterns differed between these two strains.

The observed feed conversion ratios in Atlantic salmon were about 1 and in Arctic charr were about 0.7-0.8, which are common values for Atlantic salmon (Austreng *et al.*, 1987) and Arctic charr (Jobling *et al.*, 1990), respectively.

General conclusion

Although an association between *TRP-2(92)* isozyme and growth has been established for Atlantic salmon (Torrissen, 1991; Table 6 of the present paper) and Arctic charr (Torrissen & Barnung, 1991; Table 6 of the present paper), it does not appear to be due to improved protein digestion studied by chromic oxide method. Recent experiment on studying postprandial plasma free amino acids (FAA), better absorption (higher levels of plasma FAA) and digestion (higher levels of plasma lysine) were observed in the *TRP-2(92)* salmon (Torrissen *et al.* 1992, NAVF project).

During the life cycle, there appears to be two important growth periods: The first few months after first-feeding (Torrissen *et al.* 1993) and during winter of the first sea-year (Torrissen 1991). In these two periods, the daily growth rate of salmon carrying isozyme *TRP-2(92)* was significantly higher than of those lacking the isozyme. Consequently, salmon with better daily growth rate during these two growth periods will be larger at slaughtering time, even with similar growth rate in other periods. Due to better food conversion (Torrissen & Shearer 1992), and better digestion and absorption of the dietary protein (Torrissen *et al.* 1992), the selection of trypsin *TRP-2(92)* salmon may lead to higher production, with lower feed cost, from early life until harvest.

BIOTECHNOLOGICAL ASPECT

Molecular cloning and characterization of anionic and cationic variants of trypsin from Atlantic salmon.

Abstract

Two libraries of complementary DNA to mRNA were constructed and screened with trypsin specific probes. Five clones containing near full length transcripts were selected for further characterization. The complementary DNA clones were subcloned in plasmids and sequenced in both directions. The nucleotide sequence revealed a transcript length of approximately 860 nucleotides plus a poly A tail. Northern blotting produced a band of 950 nucleotides indicating a poly A tail of near 100 nucleotides. Comparison of amino acid sequences deduced from the nucleotide sequence displayed that all variants possessed the typical serine protease active site, consisting of three amino acids; histidine, serine and aspartic acid in conserved positions. The substrate binding pocket with a trypsin determinant aspartic acid residue in the bottom is also preserved. The positions of 12 cysteine residues building 6 inter peptide bridges are likewise conserved. Translation *in vitro* of one of the trypsin clones produced a protein of expected molecular mass, 24.5 kD. Three of the Atlantic salmon trypsin clones possess very similar sequences and probably represents allelic variants coded from the same gene locus (trypsin I, IA and IB). The two other trypsin clones are probably coded from separate gene loci (trypsin II and III). Analysis of genomic DNA by southern blotting and hybridization to trypsin probe proved a complex pattern of at least 8 bands indicating a large number of gene loci for trypsin in Atlantic salmon. Distribution of charged amino acids indicates that four of the Atlantic salmon trypsin clones represents anionic forms of the enzyme while the fifth clone represents a cationic variant. Multiple alignments of the Atlantic salmon trypsin sequences with trypsin, chymotrypsin and elastase from different species proved all Atlantic salmon sequences approximately equally distant from trypsins of other species. Interestingly the distance between the anionic and cationic variants from Atlantic salmon is alike the distance between salmon and mammalian trypsin, indicating an early separation of these two types of trypsin in the evolution, possibly primal to the derivation of fish in the evolution. The Atlantic salmon nucleotide sequences has been deposited in Gene Bank/EMBL database and has recieved the following accesion numbers, trypsin I; X70075 (SSTRYPI), trypsin IA; X70071 (SSTRYPIA), trypsin IB; X7002 (SSTRYPIB), trypsin II; X70073 (SSTRYPII) and trypsin III; X70074 (SSTRYPIII).

INTRODUCTION

Trypsin, (EC 3.4.21.4) a major digestive enzyme - belong to a large family of serine proteases extensively studied in a broad range of species.

The serine proteases secreted from the acinar cells of the pancreas; chymotrypsin, trypsin and elastase are structurally very similar but demonstrates different substrate specificity due to differences in the substrate binding pocket. Trypsin is synthesized as a inactive prepro-protein, pretrypsinogen, which is activated in the intestines by enteropeptidase and/or by self activation. The maturation of pretrypsinogen starts in the pancreatic acinar cells by removal

of a N-terminal leader sequence. The resulting trypsinogen molecules are secreted to the digestive tract where enteropeptidase removes the N-terminal by specific cleavage of trypsinogen between a lysine and a isoleucine residue. The isoleucine residue now at the N-terminal of the protein bends inwards in the protein forming several attachments to residues in the internal of the protein (Bode & Huber 1986, Smalås 1990). This conformation change of the protein facilitates the formation of the catalytically active enzyme. Trypsin activates both chymotrypsin and elastase in a similar cleavage reaction. Trypsin as an endopeptidase, cuts at basic residues lysine and arginine. The catalytic site is made up by three amino acids - histidine serine aspartic acid - forming a catalytic triad which is identical in trypsin, chymotrypsin and elastase. It is the substrate binding pocket which defines the specificity of the enzymes.

Several isoforms of trypsin has been described both in mammalia and fish (Murakami & Noda 1981, Craik *et al.* 1984, Fletcher *et al.* 1987, Asgeirsson *et al.* 1989). Isoforms of trypsin may demonstrate different kinetic properties as demonstrated in the cod (Asgeirsson *et al.* 1989). However, it has also been speculated that isoforms displaying major differences in distribution of charged amino acids may have different preferences in substrate binding (Fletcher *et al.* 1987). Studies of trypsin from Atlantic salmon by isoelectric focusing on agarose gel revealed several isoforms of the enzyme (Torrissen 1984, Torrissen & Torrissen 1985). One of these trypsin isozymes denoted *TRP-2(92)* has been associated to enhanced growth (Torrissen 1987, Torrissen 1991, Torrissen *et al.* 1993) possibly through better digestion and absorption (Torrissen *et al.*, unpublished), and utilization (Torrissen & Shearer 1992) of dietary proteins.

The main objectives of this part of the project were to study the genes coding for the trypsin isozymes in Atlantic salmon with special emphasis on the variant associated to growth advantage. In further studies this could be used to gain a molecular understanding for the possible enzymatic differences and furthermore make possible development of relatively simple DNA based methods for analysis of fish demonstrating different isozyme pattern.

Methods

Chemicals and enzymes.

All cloning vectors, linkers and enzymes were purchased from Promega and New England Biolabs. Radioisotopes [α-^{35}S]dATP (1000 Ci/mmol) and [α-^{32}P]dCTP (3000 Ci/mmol) were from Amersham International. All other chemicals were purchased from Sigma if not otherwise stated.

Library construction.

RNA was extracted from Atlantic salmon pancreatic tissue according to established methods (Chirgwin *et al.* 1979, Chomczynski and Sacci 1987). The poly(A) RNA fraction was purified by two cycles of oligo(dT) column chromatography (Pharmacia). This RNA fraction was used for cDNA production by oligo(dT) (1mg/ml) priming and synthesis with RAV-2 reverse transcriptase (800 U/ml) (Amersham) according to Gubler and Hoffman (1983). The cDNA was either tailed with NotI linkers and ligated into λgt11 or EcoRI linkers when the vector λgt10 were used. To remove the excess linkers and small cDNA fragments the cDNA was size-fractionated with glass beads (Geneclean, Bio101). Subsequent cloning and packaging (Packagene, Promega) was carried out following established procedures (Sambrook *et al.* 1989). The initial λgt11 library contained only 90000 individual plaques.

The λgt10 library was estimated to contain 500000 individual plaques.

Cloning strategy

Degenerate primers derived from conserved regions in the rat trypsin; T1 and T2 (Fig. 4) (Craik *et al.* 1984, Fletcher *et al.* 1987) were used to amplify a 480 nt fragment from salmon pancreatic cDNA (see below). This trypsin fragment was subcloned and sequenced and was showen to possess extensive homology with the rat sequence. The PCR fragment was subequently used as a probe in screening the λgt11 library. Several clones were isolated. Sequencing of five clones revealed identical nucleotide sequences. Due to the relatively low number of individual clones and apparently low frequency of full length clones in the λgt11 library further screening was performed of a new λgt10 library using one of the clones (pSTRP41) from the λgt11 library as probe. Initially only truncated clones were isolated. A new probe using the 5' part of STRP41 was produced using PCR. With this probe a large number of primary clones were isolated. Based on analysis of insert length and initial sequencing four clones were selected for further characterization.

Plasmid constructs.

PCR products were gel-purified (Heery *et al.* 1990) and cloned into pGEM7zf (Promega) as described (Lorens 1991). Lambda clone inserts excised from low melting point agarose gels (BRL) and ligated directly into pGEM plasmids (Struhl 1985). The plasmid construct used in *in vitro* protein synthesis was obtained by manipulation of the pSTRP41 clone. The plasmid was opened in a unique XbaI site in the 5' polylinker and digested with S1 nuclease, blunted using Klenow fragment and finally ligated. Ten clones were isolated and sequenced. One of the clones harbouring a in frame methionine ATG codon, pSTRPdx.8, was selected for further experiments. All plasmid constructs were sequenced in both directions as ds-DNA templates with Sequenase (USB) according to the manufacturer's instructions.

Oligonuceotides.

The following oligonucleotides were purchased from MedProbe and used for sequencing or PCR experiments.

T1:	5'(A/G)TIGTI(A/T)(G/C)IGCIGCICA(C/T)TG(C/T)
T2:	5'GC(A/G)CAICC(A/G)TAICCCCAIG(A/T)IAC
STRP1.F:	5'CTATCCGGCAGCAGCAGCAA
STRP1.R:	5'CAGCTGACCATTGCACACCA
STRP2.F:	5'ACACCATGAGTTCTACCGCT
TRP5L:	5'AGCTTGATCAGCATGATGTC
TRP3F:	5'GGTGTTGTGTCCTGGGGTTA

Polymerase chain reaction, PCR.

PCR was performed on 1 - 10 ng of cDNA, λ-clones or plasmid products in a 100 µl volume containing 1X Taq buffer [10 mM Tris-HCl (pH 8.3 at 20°C), 1.5 mM $MgCl_2$, 50 mM KCl, 0.1% Triton X-100], 50 µM of each dNTP, 0.2 µM of primer, 2.5 U Taq polymerase (BRL) and run for 25 - 30 cycles (94°C, 55°C and 72°C each at 1 minute).
When the degenerated primers T1 and T2 were employed, the primer concentration was raised to 0.5 µM.

Labelling of probes.

Trypsin cDNA probe, 25 -35 ng DNA, was labelled with Klenow DNA polymerase,

employing the random priming method of Feinberg and Vogelstein (1983) using 50 - 80 µCi ^{32}P]dCTP. The specific activity of the probes were 2 - 5 x 10^9 cpm/µg. All radiolabelled probes were purified by chromatography using a Sephadex G-25 column (Pharmacia).

Standard hybridizations.

Standard hybridizations were performed at 42°C in a mixture contained 5 x SSPE, 5 x Denhardts solution, 0.25 mg/ml sonicated denatured herring sperm DNA and 0.1 % SDS. Northern blots and DNA-hybridizations using radioactive cDNA probes were conducted using a standard hybridization mixture containing 50 % formamide. After hybridization, the filters were washed 3 times for 30 min in 2 x SSPE at room temperature followed by a stringent wash at 65 °C in 0.2 x SSPE twice for 20 min.

Analysis of trypsin genes in salmon, southern blotting.

Ten µg of DNA extracted from blood samples of individual Atlantic salmon was digested with restriction enzymes. The DNA was fractionated on a 0.8 % agarose gel i TBE and blotted to a nylon filter using standard procedures (Sambrook *et al.* 1989). The filters were baked in vacuum for one hour preceding the hybridization.

RNA analysis, northern blotting.

Total RNA (10 µg) from individual Atlantic salmon were electrophoresed through a 1.1 % agarose gel containing 2 % formaldehyde and blotted to nylon filters (MSI) by simple capillary blots over night in 10 x SSPE (Sambrook *et al.* 1989). The RNA was fixed to the filters by baking at 80°C in vacuum for one hour. Hybridization was done in a standard solution.

In vitro Transcription and Translation

The plasmid pSTRP41dx.8 was used directly to program the TnT reticulocyte lysate system (Promega). The proteins were synthesized with complete amino acid mixtures. Small tracer reactions (5µl) containing 10µCi of ^{35}S-methionine were used to calculate the amount of cold protein synthesized by TCA precipitation. Aliquots of the tracer reactions (1-2µl) were analyzed on standard SDS-PAGE minigels (10%) or 15-20% gradient gels, dried and exposed to X-Ray film at room temperature for 12 hours.

Results and discussion.

Cloning and sequencing.

Two libraries of complementary DNA (cDNA) from Atlantic salmon pancreatic tissue were constructed. Initially screening were performed using a 480 nucleotides (nt) long polymerase chain reaction (PCR) generated fragment from trypsin using primers T1 and T2 from conserved regions (see Fig. 4). The PCR fragment was sequenced to confirm its identity, radiolabelled and used as a probe in the screening for trypsin sequences in a λgt11 library. A number of clones were isolated. A near full length clone (pSTRP41) was isolated and sequenced. This clone was used in further screening of the λgt11 library. However, the frequency of full length clones in the library appeared to be relatively low, consequently a new λgt10 library was constructed and screened. Totally, more than one hundred clones were isolated. Four clones determined to be near full length were chosen for further investigation. The trypsin cDNAs were subcloned into a suitable plasmid vector (pGEM7zf) and the entire

sequence determined from both strands. The results are presented in Fig. 3 A-E. All clones were truncated in the 5' end, however, two appears to contain the entire translated region (pSTRP1A and pSTRP10), two clones (pSTRP6 and pSTRP41) are truncated in the leader sequence of the protein (not present in the active trypsin) and one clone (pSTRP2) starts at amino acid residue 8 in the mature protein. The length of the clones indicates a transcript of about 860 nt plus a poly A tail. In Fig. 4 the nucleotide sequences of the five salmon trypsin clones are aligned with two trypsin sequences from rat. The pSTRP-10, -1A and -2 clones are near identical but with significant differences. However, the high identity my indicate that these cDNAs represents allele differences in the same gene locus. The pSTRP6 and pSTRP41 clones are more divergent in sequence indicating that they are coded from separate gene loci. A comparison with the nucleotide sequence of rat trypsin reveals that trypsin is relatively well conserved even at the nucleotide level through out the evolution. The identity scores are given in table 7A. The position of introns in the rat trypsin I gene (Craik *et al.* 1984) appears relatively well conserved in the salmon sequences which may indicate a similar gene structure. In Fig. 5 genomic DNA from Atlantic salmon has been analyzed by southern blotting. DNA from individual fish digested with restriction enzymes and hybridized with a STRP41 probe revealed a complex pattern of bands. In Fig. 5, digestion with EcoRI and HindIII are shown, however, similar results were obtained with a number of other restriction enzymes (results not shown). Interestingly the EcoRI digestion of DNA from individual fish in hybridization with both probes reveals a polymorfic DNA band. EcoRI does not cut any of the trypsin sequences analyzed, hence the polymorfic site is probably situated in intron and/or flanking sequences. The restriction enzyme BstEII cuts all the STRP cDNAs but clone STRP41 (Fig. 4). No polymorphies were revealed in the southern blot indicating the STRP41 locus is present in all fish examined (results not shown).

Transcription

Transcription of trypsin were analyzed by northern blotting, Fig. 6. One strong band appeared upon hybridization to a STRP41 probe. The transcript length is approximately 950 nt calculated from the migration of a RNA standard. This result is in good agreement with the length of the cDNA clones. All the trypsin mRNAs are apparently of approximately the same length. It is therefor not possible to determine the relative abundance of transcription of each variant of trypsin. In some of the lanes in Fig. 6, faint hybridization to longer transcripts can be seen. Most probably these signals are due to cross hybridization to other transcripts or possibly to non spliced or partly spliced trypsin transcripts. Strong hybridization is apparent in a smear from the main band to the bottom of the filter. This is probably due to nuclease degradation of the RNA during purification. In separate experiments using carefully purified RNA, this smear is not present, nor could any extra transcripts of increased length be detected (results not shown). The variation in hybridization signal between individual RNA samples apparent in Fig. 6, is most probably caused by variation in the amount of RNA loaded on the gel as was apparent by visualization of the RNA in the gel in UV-light after staining in ethidium bromide (results not shown). The strong hybridization signals apparent in Fig. 6 is typically from an over night exposure of the northern blot. A 20 minutes exposure was sufficient for the detection of the trypsin transcripts using 10 µg total RNA. This indicates that trypsin transcription is very strong and that trypsin transcripts may represent one percent or more of the total transcripts. As all trypsin variants examined apparently possess similar transcript lengths and are exept STRP41 highly homologous in nucleotide sequence, it is not possible to survey the relative abundancy of each transcript using Northern botting. An indication of the relative abundance of the STRP41 transcript was

obtained from experiments using PCR. Due to the relatively low identity between the nucleotide sequence of STRP41 compared to the other four trypsin clones, it was possible to construct primers for selective recognition of the STRP41 sequence (Fig. 4, results not shown). Testing of a set of primary lambda clones identified 4 as containing the STRP41 sequence compared to 18 containing the other sequences indicating that STRP41 represents approximately 1/5 of the total trypsin transcripts.

In vitro translation

The pSTRP41 clone was manipulated to obtain an initial methionine codon in frame with the trypsin sequence and used *in vitro* transcription and translation. The reaction was doped with ^{35}S-Methionine to facilitate detection of the synthesized proteins after separation by polyacrylamide gel electrophoresis. The resulting X-ray film is shown in Fig. 7. One specific band of apparent molecular mass 24.5 kD can be detected, in agreement with the expected 26.4 kD calculated from the amino acid sequence.

Amino acid sequence comparison

In Fig. 8 the amino acid sequences of the Atlantic salmon trypsins are aligned with trypsin, elastase and chymotrypsin from different species. The amino acid sequences of STRP2 and STRP1A are identical, leaving four different sequences from salmon. All trypsins have similar length. The catalytic triad histidine H57, aspartic acid D102 and serine S195 is completely conserved between all serine proteases. The amino acids generating the substrate binding pocket (specificity pocket) are of a typical trypsin nature in all salmon sequences, aspartic acid D189 in the bottom and glycines G216 and G226 lining the sides of the pocket. (The numbers refers to the classical system for chymotrypsinogen) (Hartley & Kauffman, 1966). All 12 cysteines generating 6 disulphide bonds are conserved.

Trypsin is synthesized as an inactive precursor which is activated in two steps, first by removal of a prepro sequence in the Acinar cells and secondly after secretion from the pancreas, removal of the last part of the leader sequence. The typical leader sequence is approximately 22 amino acids, the first part contain mainly hydrophobic residues determining the prepro sequence followed by a highly charged pre sequence of 9 - 10 amino acids with a typical motif of four negatively charged residues, usually one glutamic acid and three aspartic acid residues followed by a lysine. In the salmon trypsin leader sequences the overall pattern of amino acids are preserved. However, the pro sequence of trypsinogen contains only two aspartic acid residues in STRP-1A, -2, -6 and -10 plus one glutamic acid and the total length of the pre sequence appears to be 6 amino acids long. Notably, the STRP41 pre sequence contains 10 residues including four aspartic acid residues and one glutamic acid, a pattern resembling the cationic variant from rat.

From Figs. 4 and 8 and Tables 7A and B it is aparent that STRP-1A, -2 and -10 are closely related. A comparison of the mature trypsin amino acid sequences, d.e. not including the N-terminal leader, reveales that STRP-1A and -2 possess identical amino acid sequences and differs only in 3' untranslated part of the transcript. STRP10 show only one amino acid substitution compared to STRP1A and 2, STRP6 differs in 5 positions while STRP41 is differnt in 69 residues in the mature trypsin sequence. We have concluded that STRP-1A, -2 and -10 probably represents allelic differences and are coded from the same gene locus. STRP6 probably represents a sepparat gene locus as does STRP41. STRP6 is 98% identical to STRP10 (Table 7B) and possess a 3' untranslated part which is very similar to STRP2. However, the differences in the amino acid sequence is in the same range as for instance between growth hormone I and II from Atlantic salmon, which are clearly coded from

separate gene loci (Male *et al.*1992). We have therefore recomended the following naming of the trypsine sequences from Atlantic salmon, which has been adopted in Gene Bank. STRP10 is named trypsin I (Gene Bank accession number X70075, SSTRYPI), STRP1A is named trypsin IA (accession number X70071, SSTRYPIA), STRP2 is named trypsin IB (accession number X70072, SSTRYPIB), STRP6 is named trypsin II (accession number X70073, SSTRYPII) and STRYP41 is named trypsin III (accession number X70074, SSTRYPIII).

Structure and evolution

In Fig. 9 the distribution of charged amino acids in cationic and anionic variants of trypsin from rat is displayed along with STRP10 and STRP41. STRP1, 1A and 6 are very similar to STRP10 and are omitted from this comparison for the sake of clarity. The two trypsin sequences from rat diverge in charge mainly in the C-terminal part of the protein (Fletcher *et al.* 1987). The STRP10 sequence demonstrates a distribution of charged residues very similar to the anionic trypsin from rat. The net cumulative charge of trypsin summarized over the whole active trypsin is -6 for Rat anionic trypsin and +6 for Rat cationic trypsin. STRP-1A, -2, -6 and -10 variants from Atlantic salmon possess a charge of -1.5 and the STRP41 variant +4. In conclusion STRP-1A, -2, -6 and 10 all represents anionic variants of trypsin in Atlantic salmon. STRP41, however, apparently represents a cationic form of trypsin.

Fig. 10 displays the distribution of hydrophobisity in the same four trypsin sequences. It appears from the results presented in Figs. 9 and 10 that salmon and rat trypsins possess a very similar distribution of charged and hydrophobic residues, indicating a very similar tree dimensional structure. This has been confirmed from X-ray crystallographic studies of Atlantic salmon trypsin (Smalås 1990).

In Fig. 11 Atlantic salmon trypsin sequences are compared to trypsin, chymotrypsin and elastase from different species and presented as a unrooted phylogenetic tree. The analysis is based on paired alignments of all sequences investigated and thus reflects the degree of identity and not necessarily when in the evolution the separation of trypsin isoforms took place. The four isoforms of trypsin from Atlantic salmon are approximately equally distant from trypsins from mammalia, however, the node of the cationic salmon trypsin III (STRP41) appears closer connected to the mammalian sequences than the anionic trypsins of salmon. Notably the anionic and cationic trypsins from salmon are as distant as salmon versus mammalia indicating an early separation of the cationic and anionic trypsins in the evolution, possibly before the separation of fish from the rest of the animal kingdom.

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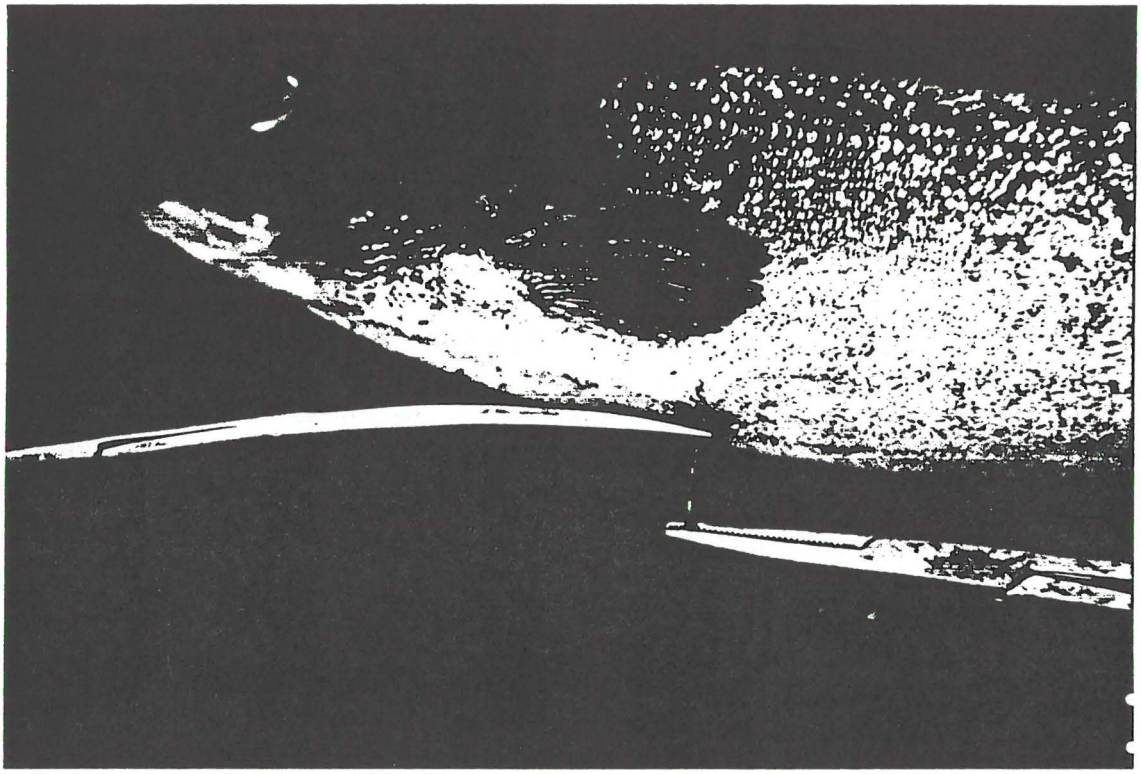

Figure 1. Biopsy technique of pyloric caeca of Atlantic salmon, showing the line cut and amount of sample removed.

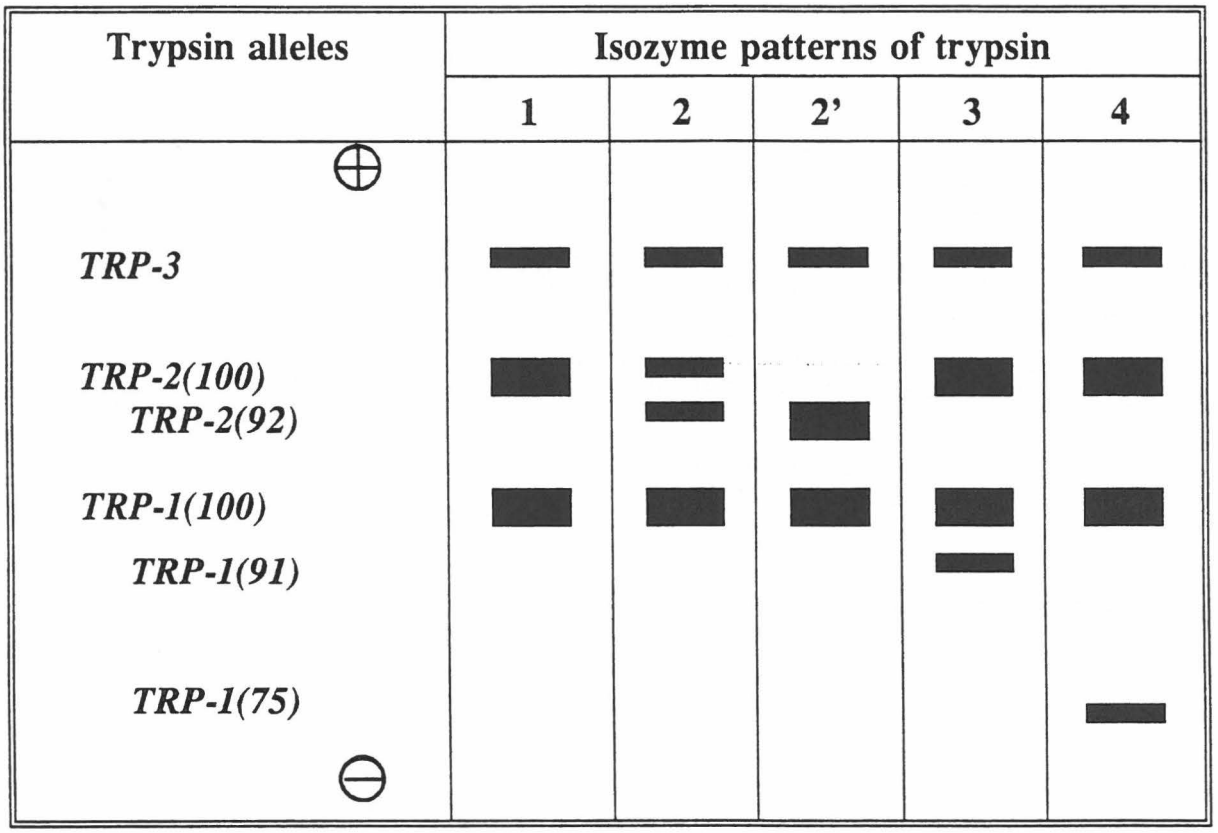

Figure 2. Trypsin isozyme patterns and designated alleles in Atlantic salmon.

```
      M  I  S  L  V  F  V  L  L  I  G  A  A  F  A  T  E  D
GCAACCATGATTTCTCTGGTCTTCGTTCTGCTCATTGGAGCCGCTTTCGCCACAGAGGAC
        10        20        30        40        50        60

 D  K  I  V  G  G  Y  E  C  K  A  Y  S  Q  T  H  Q  V  S  L
GACAAGATCGTCGGAGGGTATGAGTGCAAGGCCTACTCCCAGACCCACCAGGTGTCTCTG
        70        80        90       100       110       120

 N  S  G  Y  H  F  C  G  G  S  L  V  N  E  N  W  V  V  S  A
AACTCTGGATACCACTTCTGTGGTGGCTCCTTGGTCAATGAGAACTGGGTTGTGTCTGCT
       130       140       150       160       170       180

 A  H  C  Y  K  S  R  V  E  V  R  L  G  E  H  N  I  K  V  T
GCTCACTGCTACAAGTCCCGTGTGGAGGTGCGTCTGGGCGAGCACAACATCAAGGTGACT
       190       200       210       220       230       240

 E  G  S  E  Q  F  I  S  S  S  R  V  I  R  H  P  N  Y  S  S
GAAGGTAGCGAGCAGTTCATCTCTTCATCCCGCGTGATCCGTCACCCCAACTACAGCTCC
       250       260       270       280       290       300

 Y  N  I  D  N  D  I  M  L  I  K  L  S  K  P  A  T  L  N  T
TACAACATCGATAATGACATCATGCTGATCAAACTGAGCAAACCCGCCACCCTCAACACC
       310       320       330       340       350       360

 Y  V  Q  P  V  A  L  P  T  S  C  A  P  A  G  T  M  C  T  V
TACGTGCAGCCTGTTGCTCTGCCCACCAGCTGTGCCCCCGCTGGCACCATGTGTACCGTC
       370       380       390       400       410       420

 S  G  W  G  N  T  M  S  S  T  A  D  S  N  K  L  Q  C  L  N
TCTGGATGGGGCAACACCATGAGCTCCACTGCTGACAGCAACAAGCTGCAGTGCCTGAAC
       430       440       450       460       470       480

 I  P  I  L  S  Y  S  D  C  N  N  S  Y  P  G  M  I  T  N  A
ATCCCCATCCTGTCCTACAGCGACTGTAACAACTCCTACCCTGGCATGATCACCAACGCC
       490       500       510       520       530       540

 M  F  C  A  G  Y  L  E  G  G  K  D  S  C  Q  G  D  S  G  G
ATGTTCTGTGCTGGATACCTGGAGGGAGGCAAGGACTCTTGCCAGGGTGACTCTGGTGGC
       550       560       570       580       590       600

 P  V  V  C  N  G  E  L  Q  G  V  V  S  W  G  Y  G  C  A  E
CCTGTGGTGTGCAATGGTGAGCTCCAGGGTGTTGTGTCCTGGGGTTACGGATGTGCTGAG
       610       620       630       640       650       660

 P  G  N  P  G  V  Y  A  K  V  C  I  F  N  D  W  L  T  S  T
CCCGGTAACCCTGGTGTCTACGCCAAGGTTTGCATCTTCAATGACTGGCTGACCAGCACC
       670       680       690       700       710       720

 M  A  S  Y  *
ATGGCCTCCTACTAAGTCTGATCCTAGCTTCGGTCCTCCAGCACGGTCCCACAACTCTAC
       730       740       750       760       770       780

AACATCCCGTTCCAGATCAACATCCACCTTTTGTACGGGAGACTAGACATTATTTATGTT
       790       800       810       820       830       840

TATGATAAATAAAAAATGTAAC
       850       860
```

Figs. 3A-E Nucleotide sequence.
Nucleotide sequence of cDNA clones of Atlantic salmon trypsin with dedused amino acid sequence in the one letter code. Stop codon is indicated with asterics and poly adenylation signal is double underlined.
A Atantic salmon trypsin I. Nucleotide sequenec of cDNA clone pSTRP10 .

```
 M  I  S  L  V  F  V  L  L  I  G  A  A  F  A  T  E  D  D  K
ATGATTTCTCTGGTCTTCGTTCTGCTCATTGGAGCCGCTTTCGCCACAGAGGACGACAAG
        10         20         30         40         50         60

 I  V  G  G  Y  E  C  K  A  Y  S  Q  A  H  Q  V  S  L  N  S
ATCGTCGGAGGGTATGAGTGCAAGGCCTACTCCCAGGCCCACCAGGTGTCTCTGAACTCT
        70         80         90        100        110        120

 G  Y  H  F  C  G  G  S  L  V  N  E  N  W  V  V  S  A  A  H
GGATACCACTTCTGTGGTGGCTCCTTGGTCAATGAGAACTGGGTTGTGTCTGCTGCTCAC
       130        140        150        160        170        180

 C  Y  K  S  R  V  E  V  R  L  G  E  H  N  I  K  V  T  E  G
TGCTACAAGTCCCGTGTGGAGGTGCGTCTGGGCGAGCACAACATCAAGGTGACTGAAGGT
       190        200        210        220        230        240

 S  E  Q  F  I  S  S  S  R  V  I  R  H  P  N  Y  S  S  Y  N
AGCGAGCAGTTCATCTCTTCATCCCGCGTGATCCGTCACCCCAACTACAGCTCCTACAAC
       250        260        270        280        290        300

 I  D  N  D  I  M  L  I  K  L  S  K  P  A  T  L  N  T  Y  V
ATCGATAATGACATCATGCTGATCAAACTGAGCAAACCCGCCACCCTCAACACCTACGTG
       310        320        330        340        350        360

 Q  P  V  A  L  P  T  S  C  A  P  A  G  T  M  C  T  V  S  G
CAGCCTGTTGCTCTGCCCACCAGCTGTGCCCCCGCTGGCACCATGTGTACCGTCTCTGGA
       370        380        390        400        410        420

 W  G  N  T  M  S  S  T  A  D  S  N  K  L  Q  C  L  N  I  P
TGGGGCAACACCATGAGCTCCACTGCTGACAGCAACAAGCTGCAGTGCCTGAACATCCCC
       430        440        450        460        470        480

 I  L  S  Y  S  D  C  N  N  S  Y  P  G  M  I  T  N  A  M  F
ATCCTGTCCTACAGCGACTGTAACAACTCCTACCCTGGCATGATCACCAACGCCATGTTC
       490        500        510        520        530        540

 C  A  G  Y  L  E  G  G  K  D  S  C  Q  G  D  S  G  G  P  V
TGTGCTGGATACCTGGAGGGAGGCAAGGACTCTTGCCAGGGTGACTCTGGTGGCCCTGTG
       550        560        570        580        590        600

 V  C  N  G  E  L  Q  G  V  V  S  W  G  Y  G  C  A  E  P  G
GTGTGCAATGGTGAGCTCCAGGGTGTTGTGTCCTGGGGTTACGGATGTGCTGAGCCCGGT
       610        620        630        640        650        660

 N  P  G  V  Y  A  K  V  C  I  F  N  D  W  L  T  S  T  M  A
AACCCTGGTGTCTACGCCAAGGTTTGCATCTTCAATGACTGGCTGACCAGCACCATGGCC
       670        680        690        700        710        720

 S  Y  *
TCCTACTAAGTCTGATCCTAGCTTCGGTCCTCCAGCACGGTCCCACAACTCTACAACATC
       730        740        750        760        770        780

CCGTTCCAGATCAACATCCACCTTTTGTACGGGAGACTAGACATTATTTATGTTTATGAT
       790        800        810        820        830        840

AAATAAAAAATGTAACACTAAAAAAAAA
       850        860
```

Fig. 3B Atlantic salmon mRNA for trypsin IA.
Nucleotide sequenec of pancreas cDNA clone pSTRP1A.

```
   K  A  Y  S  Q  A  H  Q  V  S  L  N  S  G  Y  H  F  C  G  G
CAAGGCCTACTCCCAGGCCCACCAGGTGTCTCTGAACTCTGGATACCACTTCTGTGGTGG
         10         20         30         40         50         60

   S  L  V  N  E  N  W  V  V  S  A  A  H  C  Y  K  S  R  V  E
CTCCTTGGTCAATGAGAACTGGGTTGTGTCTGCTGCTCACTGCTACAAGTCCCGTGTGGA
         70         80         90        100        110        120

   V  R  L  G  E  H  N  I  K  V  T  E  G  S  E  Q  F  I  S  S
GGTGCGTCTGGGCGAGCACAACATCAAGGTGACTGAAGGTAGCGAGCAGTTCATCTCTTC
        130        140        150        160        170        180

   S  R  V  I  R  H  P  N  Y  S  S  Y  N  I  D  N  D  I  M  L
ATCCCGCGTGATCCGTCACCCCAACTACAGCTCCTACAACATCGATAATGACATCATGCT
        190        200        210        220        230        240

   I  K  L  S  K  P  A  T  L  N  T  Y  V  Q  P  V  A  L  P  T
GATCAAACTGAGCAAACCCGCCACCCTCAACACCTACGTGCAGCCTGTTGCTCTGCCCAC
        250        260        270        280        290        300

   S  C  A  P  A  G  T  M  C  T  V  S  G  W  G  N  T  M  S  S
CAGCTGTGCCCCCGCTGGCACCATGTGTACCGTCTCTGGATGGGGCAACACCATGAGCTC
        310        320        330        340        350        360

   T  A  D  S  N  K  L  Q  C  L  N  I  P  I  L  S  Y  S  D  C
CACTGCTGACAGCAACAAGCTGCAGTGCCTGAACATCCCCATCCTGTCCTACAGCGACTG
        370        380        390        400        410        420

   N  N  S  Y  P  G  M  I  T  N  A  M  F  C  A  G  Y  L  E  G
TAACAACTCCTACCCTGGCATGATCACCAACGCCATGTTCTGTGCTGGATACCTGGAGGG
        430        440        450        460        470        480

   G  K  D  S  C  Q  G  D  S  G  G  P  V  V  C  N  G  E  L  Q
AGGCAAGGACTCTTGCCAGGGTGACTCTGGTGGCCCTGTGGTGTGCAATGGTGAGCTCCA
        490        500        510        520        530        540

   G  V  V  S  W  G  Y  G  C  A  E  P  G  N  P  G  V  Y  A  K
GGGTGTTGTGTCCTGGGGTTACGGATGTGCTGAGCCCGGTAACCCCGGTGTCTACGCCAA
        550        560        570        580        590        600

   V  C  I  F  N  D  W  L  T  S  T  M  A  S  Y  *
 GGTTTGCATCTTCAATGACTGGCTGACCAGCACCATGGCCTCCTACTAAGTCTGATCCTA
        610        620        630        640        650        660

 GCTTCGGTCCTCCAGCACGGTCCCACAACTCTACAACATCCTGTGCAGTTCAATATCCAC
        670        680        690        700        710        720

 CTTATGCGCTGGACATTAAATACTAATGACAAATAAAGCATTTAACATAAAAAAAAA
        730        740        750        760        770
```

Fig. 3C Atlantic salmon mRNA for trypsin IB.
Nucleotide sequenec of pancreas cDNA clone pSTRP2.

```
   A  A  F  A  T  E  D  D  K  I  V  G  G  Y  E  C  K  A  Y  S
GAGCCGCTTTCGCCACGGAGGACGACAAGATCGTCGGAGGGTATGAGTGCAAGGCCTACT
        10         20         30         40         50         60

   Q  P  H  Q  V  S  L  N  S  G  Y  H  F  C  G  G  S  L  V  N
CCCAGCCCCACCAGGTGTCTCTGAACTCTGGGTACCACTTCTGTGGTGGCTCCTTGGTCA
        70         80         90        100        110        120

   E  N  W  V  V  S  A  A  H  C  Y  Q  S  R  V  E  V  R  L  G
ATGAGAACTGGGTTGTGTCTGCTGCTCACTGCTACCAGTCCCGTGTGGAGGTGCGTCTGG
       130        140        150        160        170        180

   E  H  N  I  Q  V  T  E  G  S  E  Q  F  I  S  S  S  R  V  I
GCGAGCACAACATCCAGGTGACTGAGGGTAGCGAGCAGTTCATCTCTTCATCCCGCGTGA
       190        200        210        220        230        240

   R  H  P  N  Y  S  S  Y  N  I  D  N  D  I  M  L  I  K  L  S
TCCGTCACCCCAACTACAGCTCCTACAACATCGACAATGACATCATGCTGATCAAGCTGA
       250        260        270        280        290        300

   K  P  A  T  L  N  T  Y  V  Q  P  V  A  L  P  T  S  C  A  P
GCAAACCCGCCACCCTCAACACCTACGTGCAGCCTGTTGCTCTGCCCACCAGCTGTGCCC
       310        320        330        340        350        360

   A  G  T  M  C  T  V  S  G  W  G  N  T  M  S  S  T  A  D  K
CCGCTGGCACCATGTGTACCGTCTCTGGATGGGGCAACACCATGAGTTCTACCGCTGACA
       370        380        390        400        410        420

   N  K  L  Q  C  L  N  I  P  I  L  S  Y  S  D  C  N  N  S  Y
AGAACAAGCTTCAGTGCCTGAACATCCCCATCCTGTCCTACAGCGACTGTAACAACTCCT
       430        440        450        460        470        480

   P  G  M  I  T  N  A  M  F  C  A  G  Y  L  E  G  G  K  D  S
ACCCTGGCATGATCACCAACGCCATGTTCTGTGCTGGATACCTGGAGGGAGGCAAGGACT
       490        500        510        520        530        540

   C  Q  G  D  S  G  G  P  V  V  C  N  G  E  L  Q  G  V  V  S
CTTGCCAGGGTGACTCCGGTGGCCCCGTGGTGTGCAATGGTGAGCTCCAGGGTGTTGTGT
       550        560        570        580        590        600

   W  G  Y  G  C  A  E  P  G  N  P  G  V  Y  A  K  V  C  I  F
CCTGGGGTTATGGCTGTGCCGAGCCCGGTAACCCCGGTGTCTACGCCAAGGTTTGCATCT
       610        620        630        640        650        660

   N  D  W  L  T  S  T  M  A  T  Y  *
TCAATGACTGGCTGACCAGCACCATGGCCACCTACTAAATCTGATCCTAGCTTCGGTCCT
       670        680        690        700        710        720

CCAGCACGGTCCCACAACACTACAACATCGTTTGCAGTTCAACATCCACCTTATGTGCTG
       730        740        750        760        770        780

GAGATTAAATACTAATGACAAATAAAGCATTTAAAATAAAAAAAAA
       790        800        810        820
```

Fig. 3D Atlantic salmon mRNA for trypsin II.
Nucleotide sequenec of pancreas cDNA clone pSTRP6.

```
 F  A  V  A  F  A  A  P  I  D  D  E  D  D  K  I  V  G  G  Y
TTCGCTGTGGCATTTGCTGCCCCCATTGACGATGAGGATGACAAGATTGTTGGAGGGTAT
        10         20         30         40         50         60

 E  C  R  K  N  S  A  S  Y  Q  A  S  L  Q  S  G  Y  H  F  C
GAGTGCAGAAAGAACTCTGCATCCTACCAGGCATCACTGCAGTCTGGCTACCACTTCTGT
        70         80         90        100        110        120

 G  G  S  L  I  S  S  T  W  V  V  S  A  A  H  C  Y  K  S  R
GGTGGCTCCCTGATCTCCAGCACATGGGTGGTGTCTGCTGCTCACTGCTACAAGTCCCGC
       130        140        150        160        170        180

 I  Q  V  R  L  G  E  H  N  I  A  V  N  E  G  T  E  Q  F  I
ATCCAGGTGCGTCTGGGTGAGCACAACATTGCCGTCAACGAGGGCACTGAGCAGTTCATT
       190        200        210        220        230        240

 D  S  V  K  V  I  M  H  P  S  Y  N  S  R  N  L  D  N  D  I
GACTCAGTAAAGGTCATCATGCACCCCAGTTACAACAGCCGCAACCTGGACAACGACATC
       250        260        270        280        290        300

 M  L  I  K  L  S  K  P  A  S  L  N  S  Y  V  S  T  V  A  L
ATGCTGATCAAGCTGAGCAAGCCCGCCTCCCTGAACAGCTATGTGAGCACTGTGGCTCTG
       310        320        330        340        350        360

 P  S  S  C  A  S  S  G  T  R  C  L  V  S  G  W  G  N  L  S
CCCTCCAGCTGTGCCAGCTCTGGCACCCGCTGTCTGGTCTCTGGCTGGGGTAACCTATCC
       370        380        390        400        410        420

 G  S  S  S  N  Y  P  D  T  L  R  C  L  D  L  P  I  L  S  S
GGCAGCAGCAGCAACTACCCCGACACTCTGAGATGCCTGGATCTCCCCATCCTGAGCAGC
       430        440        450        460        470        480

 S  S  C  N  S  A  Y  P  G  Q  I  T  S  N  M  F  C  A  G  F
AGCAGCTGCAACAGCGCCTACCCTGGACAGATCACCTCCAACATGTTCTGTGCTGGCTTC
       490        500        510        520        530        540

 M  E  G  G  K  D  S  C  Q  G  D  S  G  G  P  V  V  C  N  G
ATGGAGGGAGGCAAGGACTCTTGCCAGGGAGACTCCGGTGGCCCCGTGGTGTGCAATGGT
       550        560        570        580        590        600

 Q  L  Q  G  V  V  S  W  G  Y  G  C  A  Q  R  N  K  P  G  V
CAGCTGCAGGGTGTTGTGTCCTGGGGTTACGGCTGTGCCCAGAGGAACAAGCCTGGTGTC
       610        620        630        640        650        660

 Y  T  K  V  C  N  Y  R  S  W  I  S  S  T  M  S  S  N  *
TACACCAAGGTCTGCAACTACAGATCCTGGATCAGCAGCACCATGTCCTCCAACTAATTG
       670        680        690        700        710        720

ATCTAGACTGGAGGAGACATCTTCTCTAAGACAGAAATCCATGATAACAACTGTCATGAG
       730        740        750        760        770        780

AAAATAAATTCTATTTTACTTTTAAAAAAA
       790        800        810
```

Fig. 3E.
Atlantic salmon mRNA for trypsin III.
Nucleotide sequenec of pancreas cDNA clone pSTRP41.

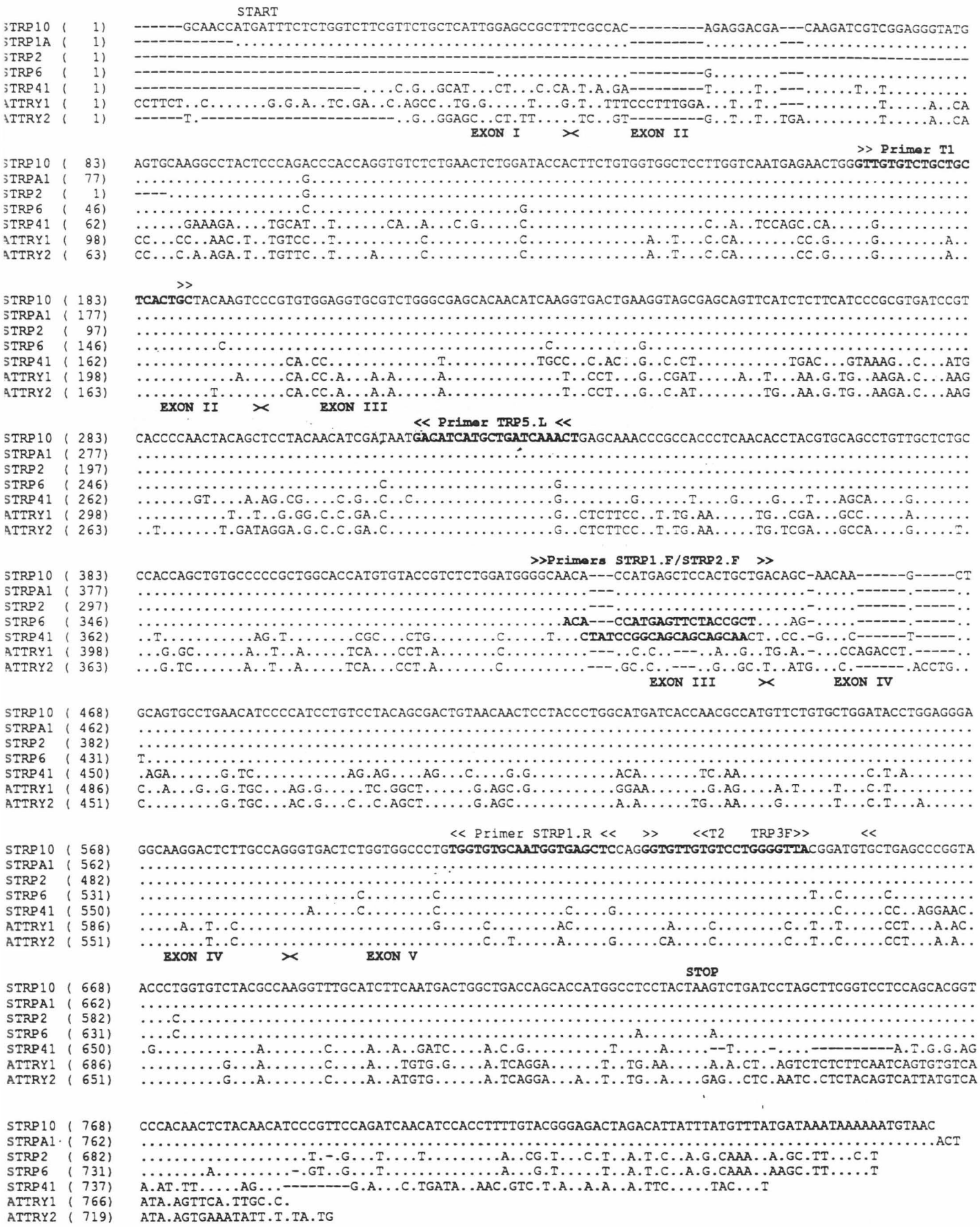

Fig. 4. Nucleotide sequence alignment.
Comparison of nucleotide sequenceses of trypsins from Atantic salmon and rat (McDonald *et al.* 1982, Craik *et al.* 1984). Identical nucleotides are indicated with a dot. Gaps are given as -. The exon/intron borders in rat trypsin I is indicated under the sequneces. Oligonucleotides used in this study are indicated.

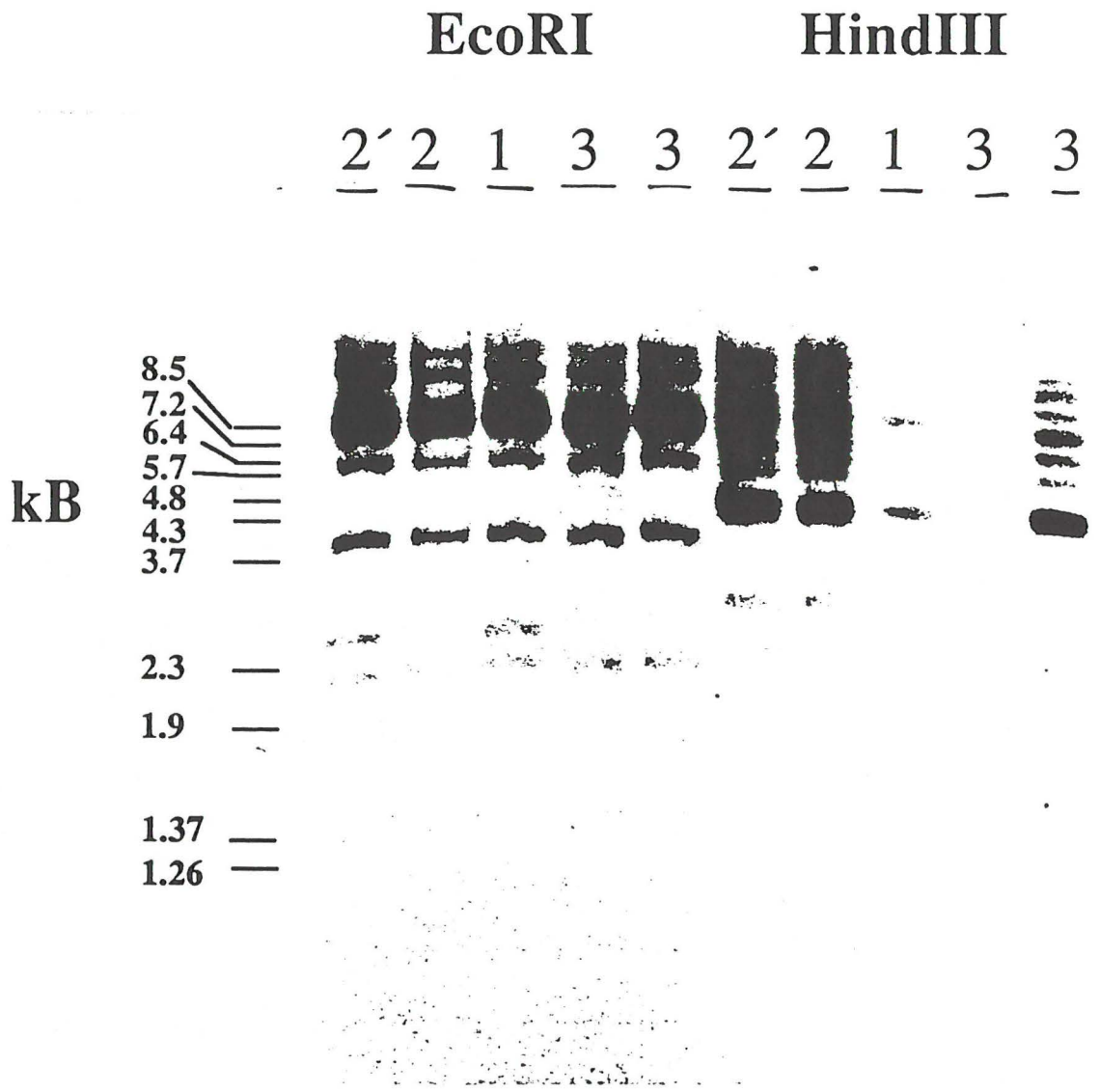

Figure 5. Southern blott.
DNA from five individual Atlantic salmon previously classified according to their trypsin isozyme pattern were examined by Southern blotting (see Fig. 1). The DNA samples were digested with restriction enzymes, fractionated on a 0.8 % agarose gel and blotted to a nylon filter (MSI) and finally hybridized to a random labelled trypsin cDNA STRP41 (see materials and methods). Each sample contained 10 µg DNA and were digested with 100 units of EcoRI or HindIII over night. The migration of a BstEII digested λ-phage DNA marker is indicated to the left.

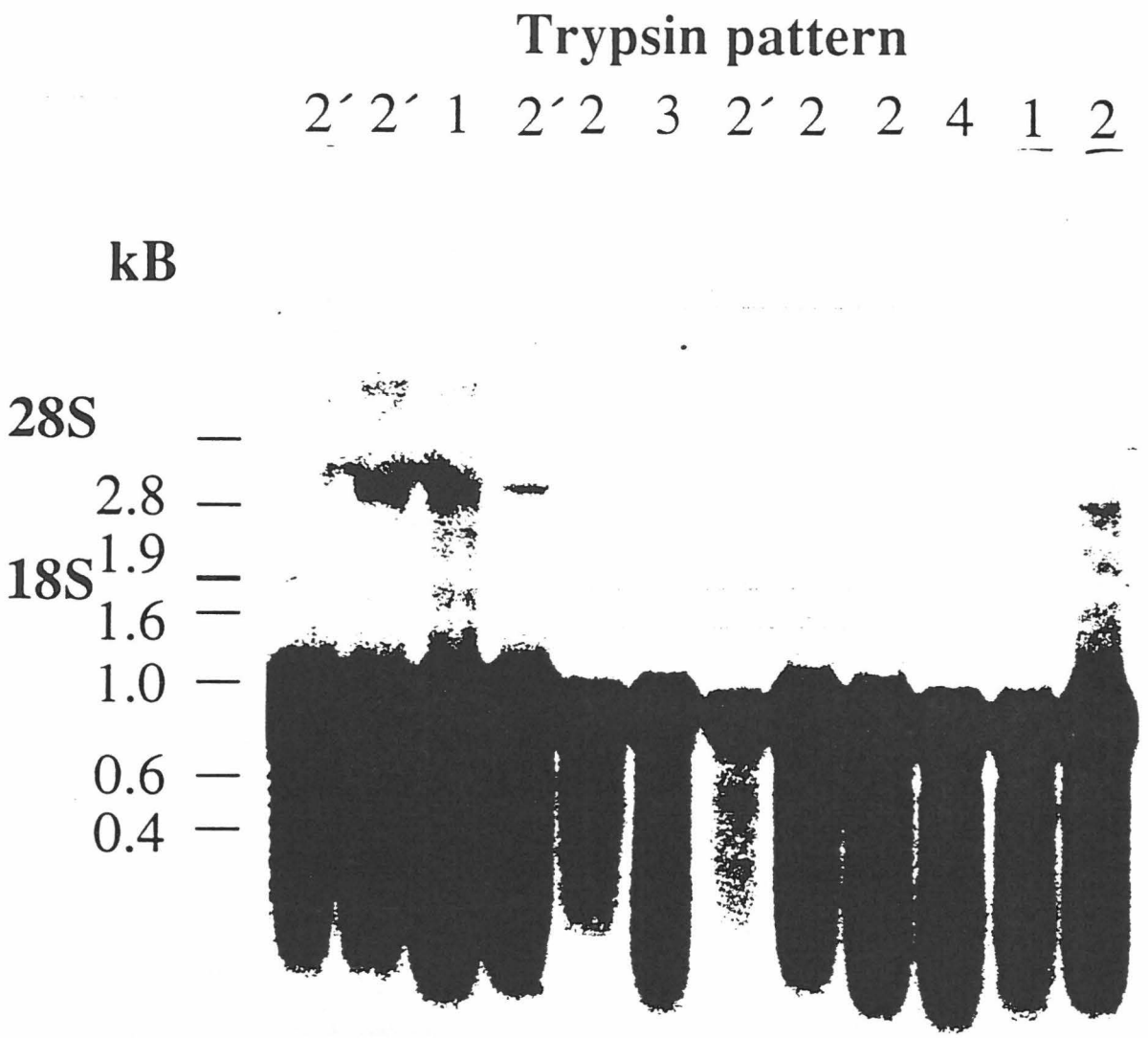

Figure 6. Northern blott.
Fractionation of RNA extracted from pancreatic tissue of individual Atlantic salmon previously assorted according to trypsin isozyme pattern (see Fig 1). Approximately 17 µg total RNA according to absorpsion at 260 nm were used in each sample. The RNA samles were treated and fractionated by agarose electrophoresis and finally blotted to a nylon membrane as indicated in materials and methods. The northern was hybrized to a STRP41 cDNA probe and exposed to XAR-5 film (Kodac) over night.

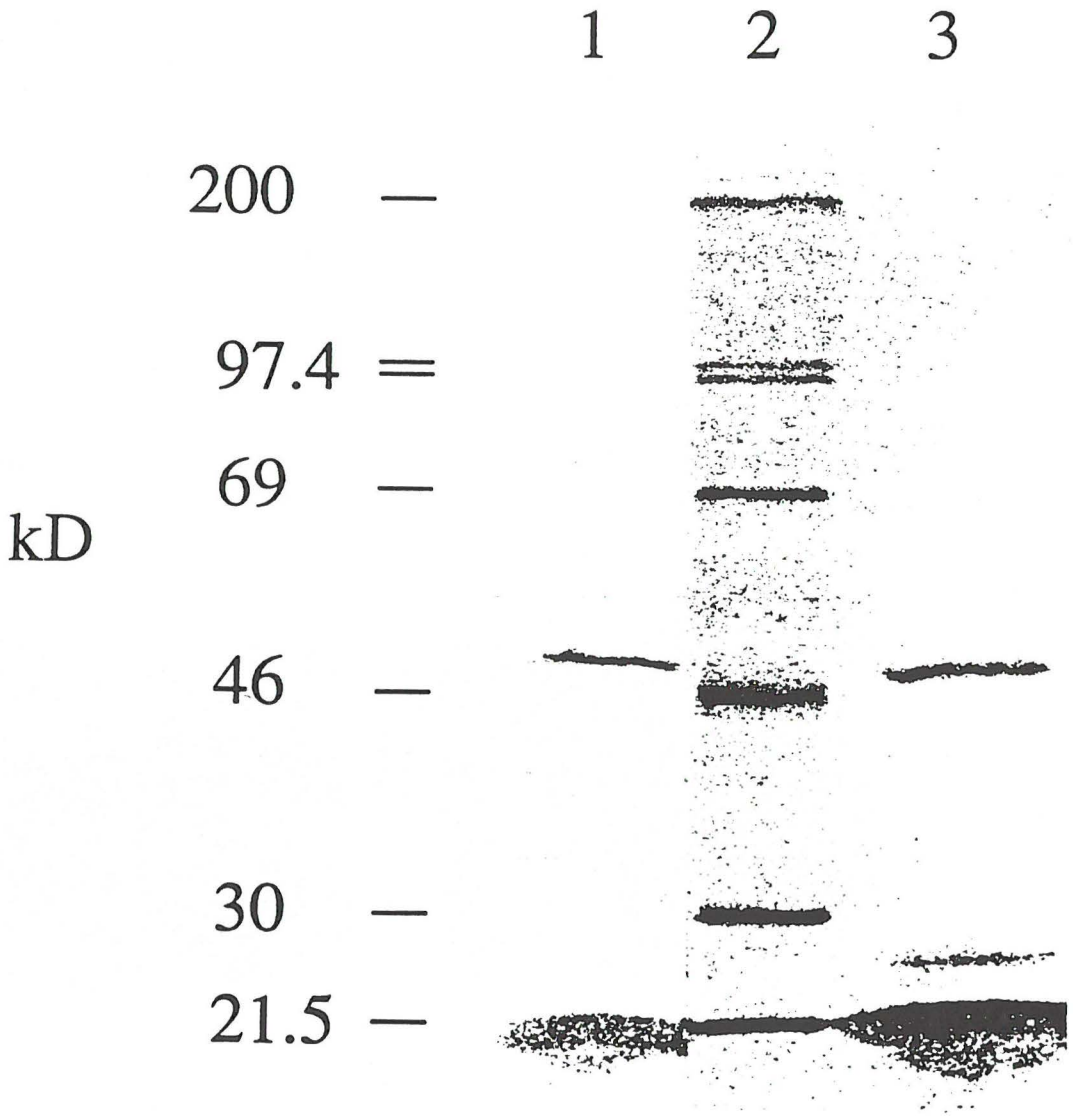

Fig. 7 ***In vitro*** **translation of salmon trypsin.**
The plasmid pSTRP41dx.8 was used directly to program the TnT reticulocyte lysate system (Promega). The protein was labelled with ^{35}S-methionine. Aliquots of the reactions were analyzed on standard SDS-PAGE minigels (12%), dried and exposed to X-Ray film at room temperature for 12 hours. Lane 1; Reaction with no RNA. Lane 2; Molecular weigth standard, ^{14}C-labeled (Amersham). Lane 3; Protein synhesized from plasmid pSTRP41dx.8.

```
                                                16              30
                                                 *   *           *
RattryIA        MSALLIL---ALVGAAVAFPLEDD----D----KIVGGYTCPEHSVPYQV
RattryIIA       MRALLFL---ALVGAAVAFPVDDD----D----KIVGGYTCQENSVPYQV
RattryIIIC      MKALIFL---AFLGAAVALPLDDD----DD---KIVGGYTCQKNSLPYQV
RattryIV        MKISIFF---AFLGAAVALPVNDD----D----KIVGGYTCPKHLVPYQV
Bovtry          ---------------------------------IVGGYTCGANTVPYQV
Dogtry          MNPLLIL---AFLGAAVATPTDDD----D----KIVGGYTCEENSVPYQV
Drotry          MLKIVIL----LSAVVCALGGTVPEGLLPQLDGRIVGGSATTISSFPWQI
mustry          MSALLIL---ALVGAAVAFPVDDD----D----KIVGGYTCRESSVPYQV
SaltrpI         MISLVFV---LLIGAAFA----TE----DD---KIVGGYECKAYSQTHQV
SaltrpIII       ------------FAVAFAAPIDDE----DD---KIVGGYECRKNSASYQA
SaltrpII        --------------AAFA----TE----DD---KIVGGYECKAYSQPHQV
SaltrpIA        MISLVFV---LLIGAAFA----TE----DD---KIVGGYECKAYSQAHQV
dogfish         A----------------P----DD----DD---KIVGGYECPKHAAPWTV
xltrp           MKFLLLC---VLLGAAAAF----D----DD---KIIGGATCAKSSVPYIV
strgr           ---------------------------------VVGGTRAAQGEFPFMV
ratkal          MPVTMWF---LILFLALSLGRNDAAPPVQS---RVVGGYNCEMNSQPWQV
elasIpig        MLRLLVVA--SLV---LYGHSTQD---FPETNARVVGGTEAQRNSWPSQI
elasIIpig       MIRALLLS--TLVAGALSCGLPAN---LPQL-PRVVGGEDARPNSWPWQV
Ratchytrp       MAFLWLVSCFALVGATFGCGVPTIQPVLTGL-SRIVNGEDAIPGSWPWQV
Dogchytrp       MAFLWLLSCFALLGTAFGCGVPAIQPVLSGL-SRIVNGEDAVPGSWPWQV
                         40                             70
                          *          *          *          *
RattryIA        SLNSGY----HF-CGGSLINDQWVVSAAHC-YKSR-IQVRLGEHNINVLE
RattryIIA       SLNSGY----HF-CGGSLINDQWVVSAAHC-YKSR-IQVRLGEHNINVLE
RattryIIIC      SLNAGY----HF-CGGSLINSQWVVSAAHC-YKSR-IQVRLGEHNIDVVE
RattryIV        SLHDGIS---HQ-CGGSLISDQWVLSAAHC-YKRK-LQVRLGEHNIHVLE
Bovtry          SLNSGY----HF-CGGSLINSQWVVSAAHC-YKSG-IQVRLGQDNINVVE
Dogtry          SLNAGY----HF-CGGSLISDQWVVSAAHC-YKSR-IQVRLGEYNIDVLE
Drotry          SLQRSGS---HS-CGGSIYSANIIVTAAHC-------LQSVSASVLQVRA
mustry          SLNAGY----HF-CGGSLINDQWVVSAAHC-YKYR-IQVRLGEHNINVLE
SaltrpI         SLNSGY----HF-CGGSLVNENWVVSAAHC-YKSR-VEVRLGEHNIKVTE
SaltrpIII       SLQSGY----HF-CGGSLISSTWVVSAAHC-YKSR-IQVRLGEHNIAVNE
SaltrpII        SLNSGY----HF-CGGSLVNENWVVSAAHC-YQSR-VEVRLGEHNIQVTE
SaltrpIA        SLNSGY----HF-CGGSLVNENWVVSAAHC-YKSR-VEVRLGEHNIKVTE
dogfish         SLNVGY----HF-CGGSLIAPGWVVSAAHC-YQRR-IQVRLGEHDISANE
xltrp           SLNSGY----HF-CGGSLITNQWVVSAAHC-YKAS-IQVRLGEHNIALSE
strgr           RLSMG---------CGGALYAQDIVLTAAHCV-SGSGNNTSITATGGVVDL
ratkal          AVYY-FG---EYLCGGVLIDPSWVITAAHC-ATDN-YQVWLGRNNLYEDE
elasIpig        SLQYRSGSSWAHTCGGTLIRQNWVMTAAHCVDRELTFRVVVGEHNLNQND
elasIIpig       SLQYDSSGQWRHTCGGTLVDQSWVLTAAHCISSSRTYRVVLGRHSLSTNE
Ratchytrp       SLQDKTG---FHFCGGSLISEDWVVTAAHCGVKTS-DVVVAGEFDQGSDE
Dogchytrp       SLQDSTG---FHFCGGSLISEDWVVTAAHCGVRTT-HQVVAGEFDQGSDA
                 80                                        110
                  *          *                *          *
RattryIA        GDEQFINAAKIIKHPNYS-----------SWTLNNDIMLIKLSSPVKLNA
RattryIIA       GDEQFINAAKIIKHPNFD-----------RKTLNNDIMLIKLSSPVKLNA
RattryIIIC      GGEQFIDAAKIIRHPSYN-----------ANTFDNDIMLIKLNSPATLNS
RattryIV        GGEQFIDAEKIIRHPEYN-----------KDTLDNDIMLIKLKSPAVLNS
Bovtry          GNQQFISASKSIVHPSYN-----------SNTLNNDIMLIKLKSAASLNS
Dogtry          GNEQFINSAKVIRHPNYN-----------SWILDNDIMLIKLSSPAVLNA
Drotry          GSTYWSSGGVVAKVSSFK-----NHEGYNANTMVNDIAVIRLSSSLSFSS
mustry          GNEQFVDSAKIIRHPNYN-----------SWTLDNDIMLIKLASPVTLNA
SaltrpI         GSEQFISSSRVIRHPNYS-----------SYNIDNDIMLIKLSKPATLNT
SaltrpIII       GTEQFIDSVKVIMHPSYN-----------SRNLDNDIMLIKLSKPASLNS
SaltrpII        GSEQFISSSRVIRHPNYS-----------SYNIDNDIMLIKLSKPATLNT
SaltrpIA        GSEQFISSSRVIRHPNYS-----------SYNIDNDIMLIKLSKPATLNT
dogfish         GDETYIDSSMVIRHPNYS-----------GYDLDNDIMLIKLSKPAALNR
xltrp           GTEQFISSSKVIRHSGYN-----------SYTLDNDIMLIKLSSPASLNA
strgr           QSAVKVRSTKVLQAPGYN-------------GTGKDWALIKLAQP------
ratkal          PFAQHRLVSQSFPHPGFNQDLIWNHTRQPGDDYSNDLMLLHLSQPADITD
elasIpig        GTEQYVGVQKIVVHPY------WNTDDVAA---GYDIALLRLAQSVTLNS
elasIIpig       PGSLAVKVSKLVVHQD------WNSNQLSN---GNDIALLKLASPVSLTD
Ratchytrp       ENIQVLKIAQVFKNPKFNM-----------FTVRNDITLLKLATPAQFSE
Dogchytrp       ESIQVLKIAKVFKNPKFNM-----------FTINNDITLLKLATPARFSK
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                  120                                                 160
                   *          *          *          *                 *
RattryIA          RVAPVALPSACA--PAGTQCLISGWGNTLSNGVNNPDLLQCVDAPVLSQA
RattryIIA         RVATVALPSSCA--PAGTQCLISGWGNTLSSGVNEPDLLQCLDAPLLPQA
RattryIIIC        RVSTVSLPRSCG--SSGTKCLVSGWGNTLSSGTNYPSLLQCLDAPVLSDS
RattryIV          QVSTVSLPRSCA--STDAQCLVSGWGNTVSIGGKYPALLQCLEAPVLSAS
Bovtry            RVASISLPTSCA--SAGTQCLISGWGNTKSSGTSYPDVLKCLKAPILSNS
Dogtry            RVATISLPRACA--APGTQCLISGWGNTLSSGTNYPELLQCLDAPILTQA
Drotry            SIKAISLATYNP--ANGASAAVSGWGTQSSGSSSIPSQLQYVNVNIVSQS
mustry            RVASVPLPSSCA--PAGTQCLISGWGNTLSNGVNNPDLLQCVDAPVLPQA
SaltrpI           YVQPVALPTSCA--PAGTMCTVSGWGNTMSSTAD-SNKLQCLNIPILSYS
SaltrpIII         YVSTVALPSSCA--SSGTRCLVSGWGNLSGSSSNYPDTLRCLDLPILSSS
SaltrpII          YVQPVALPTSCA--PAGTMCTVSGWGNTMSSTAD-KNKLQCLNIPILSYS
SaltrpIA          YVQPVALPTSCA--PAGTMCTVSGWGNTMSSTAD-SNKLQCLNIPILSYS
dogfish           NVDLISLPTGCA--YAGEMCLISGWGNTMDGAVS-GDQLQCLDAPVLSDA
xltrp             AVNTVPLPSGCS--AAGTSCLISGWGNTLSNGSNYPDLLQCLNAPILTNA
strgr             -INQPTLKIATTTAYNQGTFTVAGWGANREGGSQQRYLLK-ANVPFVSDA
ratkal            GVKVIDLPIEEP--KVGSTCLASGWGSITPDGLELSDDLQCVNIDLLSNE
elasIpig          YVQLGVLPRAGTILANNSPCYITGWGLTRTNG-QLAQTLQQAYLPTVDYA
elasIIpig         KIQLGCLPAAGTILPNNYVCYVTGWGRLQTNG-ASPDILQQGQLLVVDYA
Ratchytrp         TVSAVCLPNVDDDFPPGTVCATTGWGKTKYNALKTPEKLQQAALPIVSEA
Dogchytrp         TVSAVCLPQATDDFPAGTLCVTTGWGLTKHTNANTPDKLQQAALPLLSNA
                    170                                 200
                     *          *          *          *
RattryIA          DCEAA---YPGEITSSMICVGFLE-GGKDSCQGDSGGPVVCNG-Q----L
RattryIIA         DCEAS---YPGKITDNMVCVGFLE-GGKDSCQGDSGGPVVCNG-E----L
RattryIIIC        SCKSS---YPGKITSNMFCLGFLE-GGKDSCQGDSGGPVVCNG-Q----L
RattryIV          SCKKS---YPGQITSNMFCLGFLE-GGKDSCDGDSGGPVVCNG-E----I
Bovtry            SCKSA---YPGQITSNMFCAGYLQ-GGKDSCQGDSGGPVVCSG-K----L
Dogtry            QCEAS---YPGQITENMICAGFLE-GGKDSCQGDSGGPVVCNG-E----L
Drotry            QCASSTYGYGSQIRNTMICAA--A-SGKDACQGDSGGPLVSGG-----VL
mustry            DCEAS---YPGDITNNMICVGFLE-GGKDSCQGDSGGPVVCNG-E----L
SaltrpI           DCNNS---YPGMITNAMFCAGYLE-GGKDSCQGDSGGPVVCNG-E----L
SaltrpIII         SCNSA---YPGQITSNMFCAGFME-GGKDSCQGDSGGPVVCNG-Q----L
SaltrpII          DCNNS---YPGMITNAMFCAGYLE-GGKDSCQGDSGGPVVCNG-E----L
SaltrpIA          DCNNS---YPGMITNAMFCAGYLE-GGKDSCQGDSGGPVVCNG-E----L
dogfish           ECKGA---YPGMITNNMMCVGYME-GGKDSCQGDSGGPVVCNG-M----L
xltrp             QCNSA---YPGEITANMICVGYME-GGKDSCQGDSGGPVVCNG-Q----L
strgr             ACRSA-YG-NELVANEEICAGYPDTGGVDTCQGDSGGPMFRKDNADEWIQ
ratkal            KCVEA---HKEEVTDLMLCAGEMD-GGKDTCKGDSGGPLICNG-V----L
elasIpig          ICSSSSY-WGSTVKNSMVCAG-GD-GVRSGCQGDSGGPLHCL-VNGQYAV
elasIIpig         TCSKPGW-WGSTVKTNMICAG-GD-GIISSCNGDSGGPLNCQGANGQWQV
Ratchytrp         DCKKS---WGSKITDVMTCAG--A-SGVSSCMGDSGGPLVCQK-DGVWTL
Dogchytrp         ECKKF---WGSKITDLMVCAG--A-SGVSSCMGDSGGPLVCQK-DGAWTL
                210                                 240
                 *          *          *          *
RattryIA          QGIVSWGYG--CALPDNPGVYTKVCNFVGWIQDTIAAN
RattryIIA         QGIVSWGYG--CALPDNPGVYTKVCNYVDWIQDTIAAN
RattryIIIC        QGVVSWGYG--CAQKGKPGVYTKVCNYVNWIQQTVAAN
RattryIV          QGIVSWGSV--CAMRGKPGVYTKVCNYLSWIQETMANN
Bovtry            QGIVSWGSG--CAQKNKPGVYTKVCNYVSWIKQTIASN
Dogtry            QGIVSWGYG--CAQKNKPGVYTKVCNFVDWIQSTIAANS
Drotry            VGVVSWGYG--CAYSNYPGVYADVAVLRSWVVSTANSI
mustry            QGIVSWGYG--CAQPDAPGVYTKVCNYVDWIQNTIADN
SaltrpI           QGVVSWGYG--CAEPGNPGVYAKVCIFNDWLTSTMASY
SaltrpIII         QGVVSWGYG--CAQRNKPGVYTKVCNYRSWISSTMSSN
SaltrpII          QGVVSWGYG--CAEPGNPGVYAKVCIFNDWLTSTMATY
SaltrpIA          QGVVSWGYG--CAEPGNPGVYAKVCIFNDWLTSTMASY
dogfish           QGIVSWGYG--CAERDHPGVYTRVCHYVSWIHETIASV
xltrp             QGVVSWGYG--CAMRNYPGVYTKVCNYNAWIQNTIAAN
strgr             VGIVSWGYG--CARPGYPGVYTEVSTFASAIASAARTL
ratkal            QGITSWGFN-PCGEPKKPGIYTKLIKFTPWIKEVMKENPS
elasIpig          HGVTSFVSRLGCNVTRKPTVFTRVSAYISWINNVIASN
elasIIpig         HGIVSFGSSLGCNYYHKPSVFTRVSNYIDWINSVIANN
Ratchytrp         AGIVSWGSG-VCSTST-PAVYSRVTALMPWVQQILEAN
Dogchytrp         VGIVSWGSG-TCSTST-PGVYARVTKLIPWVQQILQAN
```

Fig 8. **Alignment of amino acid sequences of serine proteases.**
The sequences were obtained from the Gene Bank or Swiss prot databases. For references see Fig. 11. The sequences has been numberd according to chymotrypsin (Hartley & Kaufmann 1966).

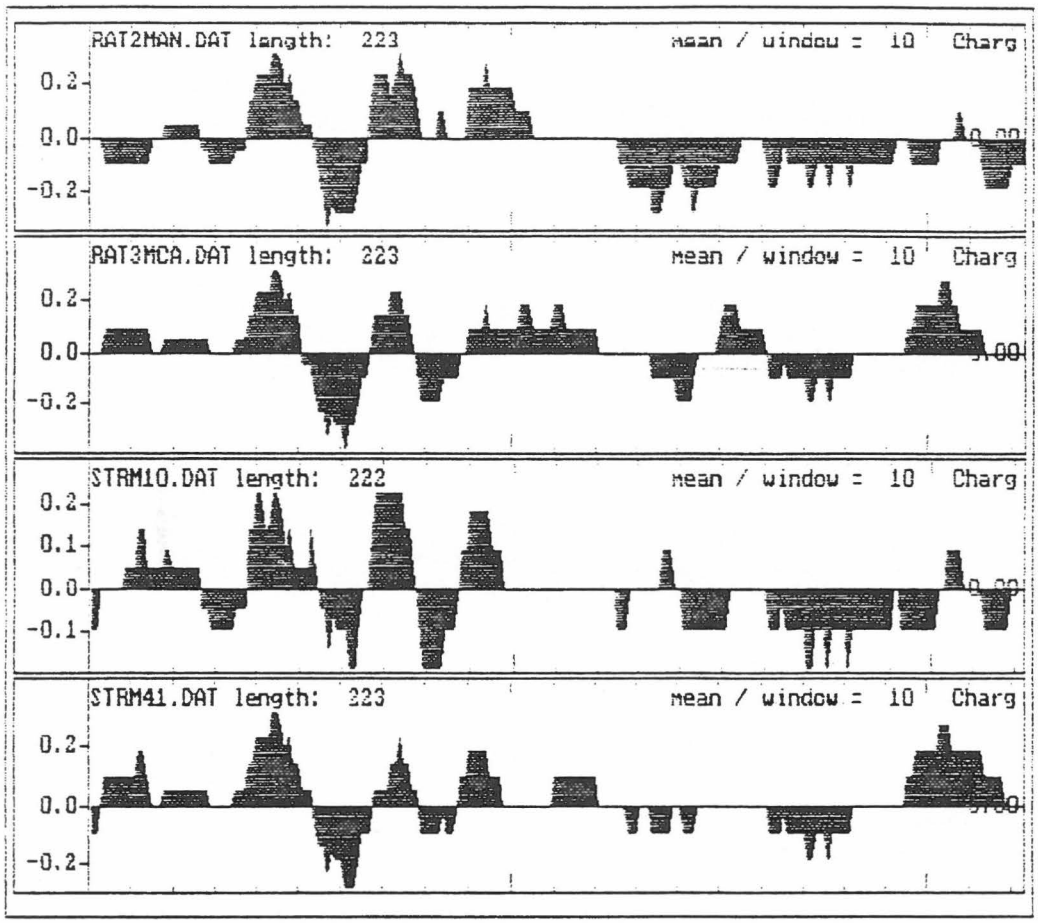

Fig. 9. Distribution of charged amino acids in trypsin.
Analysis of rat trypsin 1; anionic variant (McDonald *et al.* 1982), rat trypsin 3; cationic variant (Fletcher *et al.* 1987), salmon trypsin I (STRPM10) and salmon trypsin III (STRPM41) using the computer program PROFILEGRAPH. Charge of amino acids are plotted using a 10 reside window. Only the amino acids of mature trypsin are included.

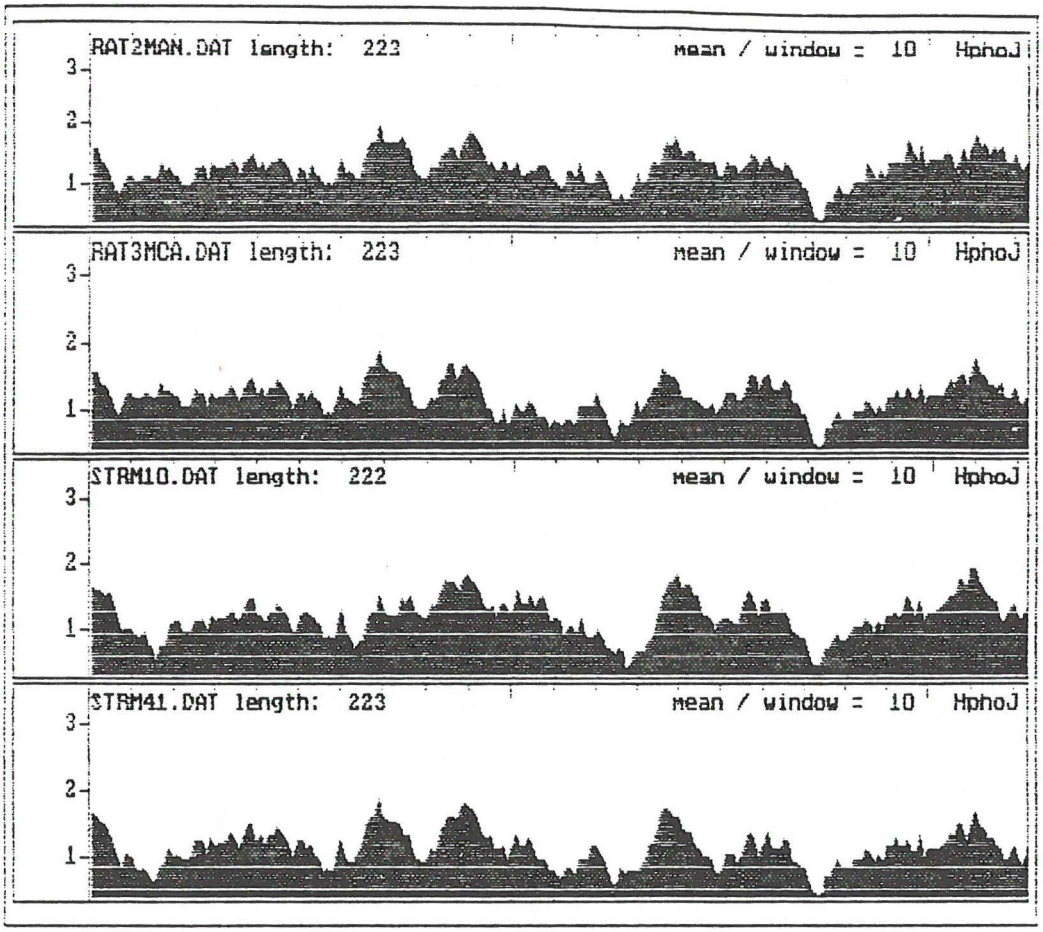

Fig. 10. Distribution of hydrophoc residues in trypsin.
Analysis of rat trypsin 1; anionic variant (McDonald *et al.* 1982), rat trypsin 3; cationic variant (Fletcher *et al.* 1987), salmon trypsin I (STRPM10) and salmon trypsin III (STRPM41) using the computer program PROFILEGRAPH. The hydrophobicity score of Jones (1975) has been employed and dispayed using a 10 residue window. Only the amino acids of mature trypsin are included.

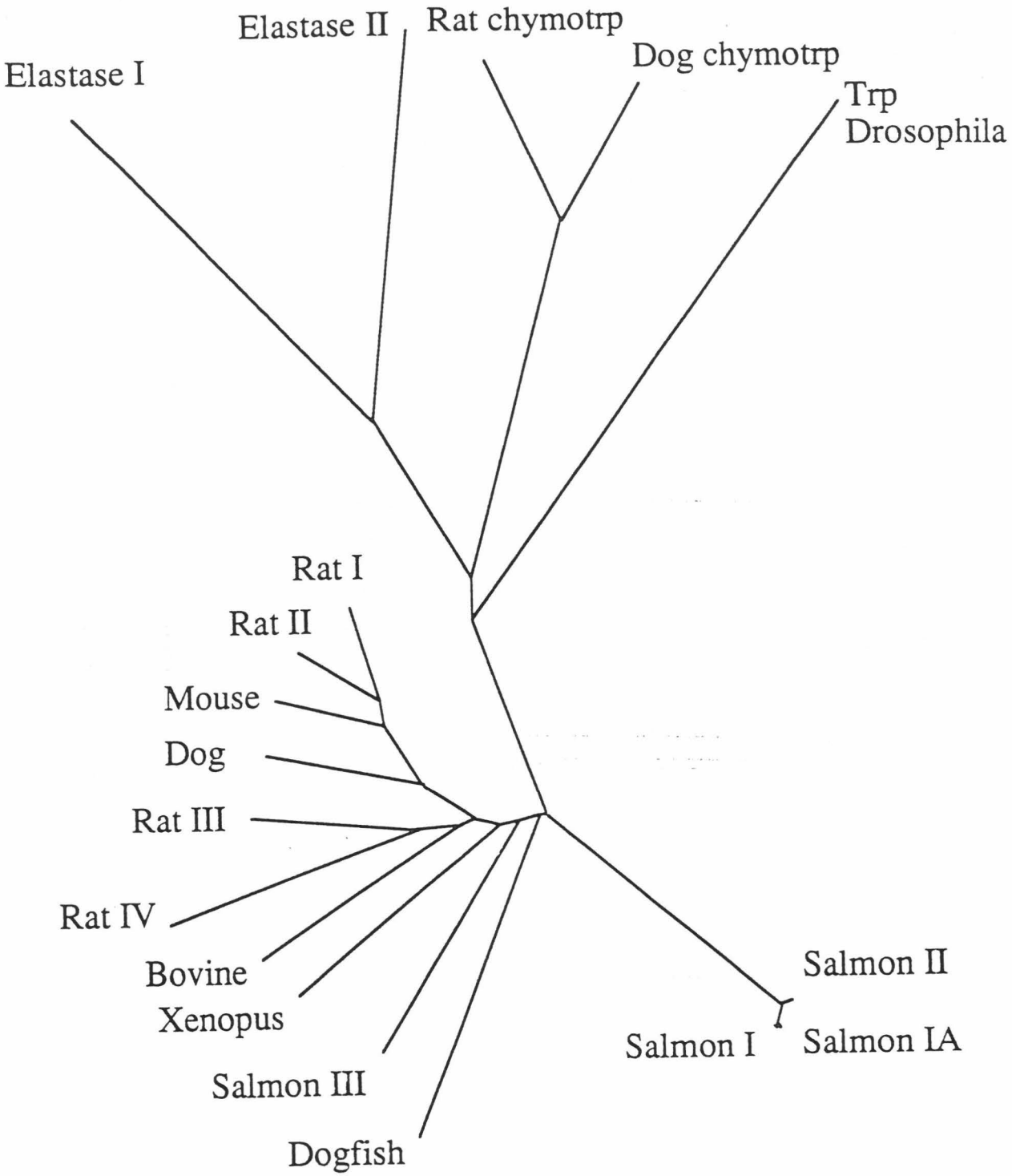

Fig. 11. Phylogenetic tree of serine proteases.
An unrooted phylogenetic tree drawn by the neighbor-joining method (Saitou & Nei, 1987) based on alignment of amino acid sequences as shown in Fig. 8 using the CLUSTAL computer program (Higgins & Sharp 1988). The figure is drawn to scale; the distance indicating relative divergence. The sequences were obtained from the Gene Bank or Swiss prot databases. Rat I; rat anionic trypsin I (McDonald *et al.* 1982), Rat II; rat anionic trypsin II (McDonald *et al.* 1982, Craik *et al.* 1984), Rat III; rat cationic trypsin III (Fletcher *et al.* 1987), Rat IV; rat trypsin IV (Luetcke *et al.* 1989), Bovine; bovine trypsin (Le Huerou 1990), Dog; dog anionic trypsin (Pinsky *et al.* 1985), Trp Drosophila; *D.melanogaster* trypsin-like enzyme (Davis *et al.* 1985), Mouse; mouse trypsin (Stevenson 1986), Dogfish; dogfish trypsin (Hermodson *et al.* 1971, Titani *et al.* 1975), Xenopus; *Xenopus Laevis* trypsin (Shi & Brown 1990), Elastase I; porcine elastase I (Shirasu *et al.* 1986), Elastase II; porcine elastase II (Kawashima *et al. 1987),* Rat chymotrp; rat chymotrypsin B (Bell *et al.* 1984) and Dog chymotrp; dog chymotrypsin (Pinsky *et al.* 1983).

Table 1. Heredity study of the polymorphic trypsin locus *TRP-1* showing apparent phenotype distribution of the progeny and the estimated parental genotype. The observed parental genotypes are shown in Table 2. The estimated parental genotypes present the alleles that were observed in the offspring but do not indicate either disomic or tetrasomic inheritance. (Parental genotypes with the same superscript are the same fish, and the frequencies in parentheses are possibly error.)

Family	N	Estimated parental *TRP-1* genotypes		Apparent phenotypic frequency distributions in the offspring				
		Female	Male	*TRP-1 (100/100)*	*TRP-1 (100/91)*	*TRP-1 (91/91)*	*TRP-1 (91/75)*	*TRP-1 (100/75)*
1	71	*100/100/100/91*	*100/100/100/91*	0.89	0.11	-	-	-
2	39	[a] *100/100/91/91*	[h] *100/100/91/91*	0.28	0.67	0.05	-	-
3	65	[b] *100/100/100/91*	*100/100/91/91*	0.46	0.52	-	-	(0.02)
4	46	[c] *100/100/100/91*	*100/100/75/75*	0.59	-	-	0.02	0.39
5	64	*100/100/75/75*	*100/100/100/91*	0.45	0.06	-	-	0.49
6	47	[c] *100/100/100/91*	[i] *100/100/100/100*	0.96	0.04	-	-	-
7	57	*100/100/100/75*	*100/100/100/75*	0.46	-	-	-	0.54
8	48	[a] *100/100/91/91*	*100/100/100/91*	0.60	0.40	-	-	-
9	55	[a] *100/100/91/91*	[k] *100/100/75/75*	0.31	0.15	-	0.16	0.38
10	60	*100/100/100/91*	[l] *100/100/100/91*	0.97	0.03	-	-	-
11	69	[b] *100/100/100/91*	[m] *100/100/75/75*	0.64	0.01	-	-	0.35
12	39	[d] *100/100/100/91*	[h] *100/100/91/91*	0.59	0.41	-	-	-
13	64	*100/100/100/91*	*100/100/75/75*	0.54	0.05	-	0.05	0.36
14	23	*100/100/100/91*	*100/100/100/100*	0.96	0.04	-	-	-
15	53	*100/100/100/91*	[k] *100/100/75/75*	0.51	0.07	-	-	0.42
16	61	[e] *100/100/100/75*	*100/100/75/75*	0.41	-	-	-	0.59
17	54	[d] *100/100/100/91*	[i] *100/100/100/100*	0.93	0.07	-	-	-
18	53	[f] *100/100/100/100*	[k] *100/100/75/75*	0.77	(0.02)	-	-	0.21
19	62	*100/100/100/75*	[l] *100/100/100/91*	0.95	-	-	-	0.05
20	66	[e] *100/100/100/75*	[m] *100/100/75/75*	0.42	-	-	-	0.58
21	61	*100/100/100/100*	[l] *100/100/100/91*	0.92	0.08	-	-	-
22	54	[f] *100/100/100/100*	*100/100/100/100*	1.00	-	-	-	-
23	44	[f] *100/100/100/100*	*100/100/100/100*	1.00	-	-	-	-
24	60	*100/100/100/75*	*100/100/100/75*	0.63	-	-	-	0.37
25	58	[g] *100/100/100/91*	*100/100/100/91*	0.81	0.19	-	-	-
26	51	[g] *100/100/100/91*	*100/100/75/75*	0.49	0.14	-	-	0.37

Table 2. Heredity study of the polymorphic trypsin locus *TRP-2* showing apparent phenotype distribution of the progeny and the estimated parental genotype. The estimated parental genotypes present the alleles that were observed in the offspring but do not indicate either disomic or tetrasomic inheritance. (Parental genotypes with the same superscript are the same fish.)

Family	N	Observed isozyme pattern		Estimated parental *TRP-2* genotypes		Apparent phenotypic frequency distributions in the offspring		
		Female	Male	Female	Male	*TRP-2* (100/100)	*TRP-2* (100/92)	*TRP-2* (92/92)
1	71	1	1	*100/100/100/100*	*100/100/100/100*	1.00	-	-
2	39	3	3	[a] *100/100/100/92*	[h] *100/100/100/92*	1.00	-	-
3	65	1	3	[b] *100/100/100/92*	*100/100/100/100*	1.00	-	-
4	46	1	4	[c] *100/100/100/92*	*100/100/100/100*	0.91	0.09	-
5	64	4	2	*100/100/100/92*	*100/100/92/92*	0.45	0.52	0.03
6	47	1	2	[c] *100/100/100/92*	[i] *100/100/92/92*	0.62	0.21	0.17
7	57	1	2'	*100/100/100/92*	*100/92/92/92*	0.61	0.37	0.02
8	48	3	2	[a] *100/100/100/92*	*100/100/92/92*	0.58	0.25	0.17
9	55	3	2+4	[a] *100/100/100/92*	[k] *100/92/92/92*	0.39	0.09	0.52
10	60	3	2+3	*100/100/100/92*	[l] *100/92/92/92*	0.37	0.42	0.21
11	69	1	2'+4	[b] *100/100/100/92*	[m] *100/92/92/92*	0.35	0.32	0.33
12	39	2	3	[d] *100/92/92/92*	[h] *100/100/100/92*	0.33	0.31	0.36
13	64	3	2+4	*100/100/100/92*	*100/100/92/92*	0.33	0.59	0.08
14	23	3	2'	*100/100/100/92*	*100/92/92/92*	0.31	0.39	0.30
15	53	1	2+4	*100/100/100/92*	[k] *100/92/92/92*	0.30	0.28	0.42
16	61	2	4	[e] *100/100/92/92*	*100/100/100/92*	0.28	0.43	0.29
17	54	2	2	[d] *100/92/92/92*	[i] *100/100/92/92*	0.26	0.35	0.39
18	53	2	2+4	[f] *100/100/92/92*	[k] *100/92/92/92*	0.23	0.42	0.35
19	62	2	2+3	*100/100/92/92*	[l] *100/92/92/92*	0.13	0.26	0.61
20	66	2	2'+4	[e] *100/100/92/92*	[m] *100/92/92/92*	0.12	0.61	0.27
21	61	1	2+3	*100/100/100/92*	[l] *100/92/92/92*	0.11	0.41	0.48
22	54	2	2	[f] *100/100/92/92*	*100/100/92/92*	0.09	0.39	0.52
23	44	2	2'	[f] *100/100/92/92*	*100/92/92/92*	0.07	0.30	0.63
24	60	2	2'	*100/100/92/92*	*100/92/92/92*	0.05	0.33	0.62
25	58	2'	3	[g] *92/92/92/92*	*100/100/100/92*	-	0.52	0.48
26	51	2'	4	[g] *92/92/92/92*	*100/100/100/92*	-	0.41	0.59

Table 3. Average weights of salmon with and without the trypsin isozyme *TRP-2(92)* from three different populations, at the start and at the end of the experiment.

Salmon strain	Weight±SEM (g), October 90		Weight±SEM (g), February 91	
	With	Without	With	Without
Dale (f=0.29)	$52.7\pm2.2_a$	$42.6\pm1.2_b$	$64.3\pm2.9_a$	$50.2\pm1.5_b$
Lonevåg (f=0.10)	$47.9\pm2.8_a$	$40.7\pm0.8_b$	*55.0±4.0	*47.5±1.0
Voss (f=0.09)	$40.3\pm1.7_b$	$40.5\pm0.7_b$	$51.2\pm2.0_b$	$49.0\pm0.7_b$

f=Frequency of the isozyme *TRP-2(92)* in each strain.
Means of the same period with different subscripts (a and b), or with the same sign *, are significant different ($P<0.04$).

Table 4. Apparent digestibility coefficient (ADC) of protein and specific growth rate (SGR) of Atlantic salmon smolts with and without the variant *TRP-2(92)* and of two strains of Arctic charr. Average temperature was about 6°C.

Fish group	Average weight ± sem (g)		SGR	ADC of protein
	Initial	Final	(% per day)	(%)
Atlantic salmon				
With *TRP-2(92)* ($n = 3$)	58.0±0.7	72.9±0.7	0.23±0.02	81.8*
Without *TRP-2(92)* ($n = 5$)	49.0±0.7	58.7±1.3	0.18±0.02	82.7*
Arctic charr				
"Hammerfest" strain ($n = 4$)	87.7±3.0	113.9±4.1	0.26±0.03	82.9±1.2
"Skogseid" strain ($n = 4$)	33.0±0.9	37.3±0.9	0.12±0.04	80.6±0.2

(* No replication)

Table 5. Apparent digestibility coefficient (ADC) of protein and specific growth rate (SGR) of Atlantic salmon post-smolts with and without the variant *TRP-2(92)*. Average temperature was about 10 °C.

Isozyme group	ADC of protein (%)				SGR
	Day 7	Day 14	Day 21	Day 28	(% per day)
With *TRP-2(92)*					
1	79.2	81.2	80.8	79.6	0.95
2	80.4	81.3	81.8	83.4	0.70
3	79.8	81.5	80.3	78.5	0.73
4	81.7	78.8	79.4	82.7	0.74
5	83.1	83.3	81.1	82.1	0.78
6	81.6	82.0	80.7	80.9	0.90
Mean±sem	81.0 ±0.6	81.4 ±0.6	80.7 ±0.3	81.2 ±0.8	0.80 ±0.04
Without *TRP-2(92)*					
1	82.3	82.8	81.9	82.4	0.80
2	80.7	82.2	79.6	83.0	0.79
3	80.5	81.0	78.9	81.5	0.69
4	83.5	80.2	81.1	82.1	0.61
Mean±sem	81.7 ±0.7	81.6 ±0.6	80.4 ±0.7	82.2 ±0.3	0.72 ±0.05

Table 6. Feed utilization in Atlantic salmon smolts, groups with and without *TRP-2(92)* and in two strains of Arctic charr. Average temperature was about 6 °C.

Fish group	Total weight ± sem (g)		SGR	FCR	PER
	Initial	Final	(% per day)		
Atlantic salmon					
With *TRP-2(92)* (n = 3)	3350±58	4151±64	0.39±0.01 (n=130)	0.89±0.01	2.2±0.0
Without *TRP-2(92)* (n = 5)	3187±46	3895±64	0.37±0.01 (n=253)	0.99±0.02	1.9±0.0
	P=0.07	P=0.04	P=0.02	P=0.02	P=0.01
Arctic charr					
"Hammerfest" strain (n = 2)	7971±22	10188±114	0.69±0.04 (n=84)	0.66±0.04	2.9±0.2
"Skogseid" strain (n = 1)	7752	9534	0.52±0.07 (n=121)	0.82	2.3
			P=0.02		

A.

Reference molecule:		pSTRP10	1 - 862	(862 bps)	Homology
Sequence	2:	pSTRP1A	1 - 868	(868 bps)	99%
Sequence	3:	pSTRP2	1 - 777	(777 bps)	87%
Sequence	4:	pSTRP6	1 - 826	(826 bps)	90%
Sequence	5:	pSTRP41	1 - 810	(810 bps)	68%
Sequence	6:	RATTRY1	1 - 804	(804 bps)	64%
Sequence	7:	RATTRY2	1 - 773	(773 bps)	62%

Parameters set: Mismatch = 2; Open Gap = 4; Extend Gap = 1

B

		Name	Region	Length	Identical residues	Homology
Reference molecule:		Trypsin I	21 - 242	(222 aa)	-	
Sequence	2:	Trypsin IA	21 - 242	(222 aa)	221	100%
Sequence	3:	Trypsin IB	1 - 215	(215 aa)	214	96%
Sequence	4:	Trypsin II	10 - 231	(222 aa)	218	98%
Sequence	5:	Trypsin III	16 - 238	(223 aa)	154	69%
Sequence	6:	Rattryp I	24 - 246	(223 aa)	148	67%
Sequence	7:	Rattryp II	16 - 238	(223 aa)	147	67%

Parameters set: Mismatch = 2; Open Gap = 4; Extend Gap = 1

Table 7 Identity between trypsins from Atlantic salmon and rat.
Computer alignments were performed using the ALIGN program (Scientific and Educational Software).
A: Comparison of nucleotide sequences of trypsin variants from Atlanic salmon and two trypsin sequences from rat to the trypsin clone pSTRP10. The rat sequences were obtained from the EMBL database, Rattry1 (McDonald *et al.* 1982), Rattry2 (McDonald *et al.* 1982, Craik *et al.* 1984).
B: Comparison of amino acid sequences of trypsin variants from Atlantic salmon and rat to salmon trypsin I. Only the sequence of the active trypsin is used, d.e. the N-terminal leader has been omitted.