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냉수성 고유어종의 생식선 자극 호르몬 cDNA의 cloning 및
단백질 대량생산

(Cloning of gonadotropin cDNAs and its expression in salmonids and
native fishes to korea)

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제 출 문

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본 보고서를 “냉수성 고유어종의 생식선 자극 호르몬 cDNA의 cloning 및 단백질 대량생산” 과제의 최종보고서로 제출합니다.

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요 약 문

I. 과 제 명

냉수성 고유어종의 생식선 자극 호르몬 cDNA의 cloning 및 단백질 대량생산

II. 연구개발의 목적 및 필요성

1. 연구의 목적

가. 강원도 고유의 냉수성 어종의 멸종을 방지하고 번식시키기 위함

나. 생명 공학적 연구 방법을 검토하기 위하여 냉수성 고유 어종의 생식선 자극 호르몬 cDNA를 cloning하고 단백질을 대량 생산하고자 함

2. 연구 개발의 기술·경제·사회적 필요성

가. 북미와 유럽 특히 미국의 대규모화된 기업형 양식가들은 자국정부의 방조에 가까운 유전자 재조합 농산물(GMO)의 확대 생산에 힘입어 저가의 수산물을 안정적으로 수출 할 길을 모색하고 있으며, 중국은 값싼 노동력과 풍부한 수산물을 배경으로 수출한 결과 우리 어민의 생계에 치명적인 타격을 주고 있는 실정임

나. 국민의 식생활 기호에 맞는 순수한 고유 어종을 보존하고 번식시켜 양식 산업화시키는 것은 WTO체제에서 우리 어민이 생존할 수 있는 길의 하나라고 판단됨

다. 산업 기반 시설이 빈약한 강원도지역의 특성을 고려할 때 국내 양식 산업을 새로 주도할 수 있는 메카로서의 재도약이 기대됨

III. 연구 개발의 내용 및 범위

1. 연구 개발의 최종 목표: 생명 공학을 통한 강원도 특산 어종의 번식 제어

2. 일차 년도 : 생식선 자극 호르몬 cDNA의 cloning

3. 이차 년도 : 호르몬의 인위적 대량 생산

4. 삼차 년도 : 호르몬의 활성을 생체 내에서 확인

IV. 연구개발결과

1. 생식선자극호르몬의 cloning

포유류에서 하등척추동물인 어류까지 생식선자극호르몬 (GTH)는 2종류가 있음이 알려져 있다. 즉, 성성숙의 초기단계에서 작용하는 FSH와 최종성숙단계에서 작용하는 LH가 있다. 이들 각각의 호르몬은 어종 간에 30-90%의 유사성

을 가지고 있는 특징을 이용하여 cloning하였다.

- 가. 강원도 고유의 냉수성 어류 또는 천연 기념물급 어류를 선정하고 확보하여 뇌하수체를 적출한 후 급속냉동보존(-80°C)
- 나. 뇌하수체에서 추출한 total RNA 또는 mRNA를 출발 재료로 하여 Oligo dT primer와 역전사효소를 사용하여 역전사 반응을 *in vitro*에서 시킨 후 대상 어종의 뇌하수체 cDNA pool을 확보
- 다. 보고된 어류의 FSH β 및 LH β 와 가장 유사한 부위의 염기 배열을 도출하여 합성 Oligo primer를 설계
- 라. 뇌하수체 cDNA pool을 template로 하여 PCR방법으로 FSH β 및 LH β 의 cDNA를 증폭하고 cloning
- 마. FSH 및 LH의 공통적인 subunit인 α -subunit은 동일한 방법으로 cloning함

2. 호르몬의 인위적 대량 생산

- 가. cloning화된 α subunit 1분자와 FSH β 또는 LH β 1분자를 진핵 생물에서 강제적으로 발현시킬 수 있는 vector에 삽입
- 나. 발현 상황은 SDS-PAGE방법으로 확인
- 다. 발현 밴드를 확인 후 항원-항체반응을 이용한 Western blot 분석법으로 유전자재조합 FSH 및 LH의 재확인
- 라. 대량 생산할 수 있는 최적 조건의 검토 및 Affinity column을 이용하여 정제

3. 호르몬의 활성을 생체 내에서 확인

- 가. 실험 대상 어류의 생식선 발달 초기에 FSH를 복강 투여 3일 후 혈액과 생식소 sampling
- 나. 채취한 혈장으로부터 스테로이드호르몬의 측정 결과 FSH를 투여한 그룹에서 에스트라디올(E2)이 현저히 증가함
- 다. 생식소를 조직학적으로 관찰한 결과 대조군에 비하여 난모 세포가 현저히 발달(난황이 축적된 과립구의 형성 촉진, 난황 형성기의 중기에 도달한 난모 세포 다수 출현)

V. 연구 개발 결과의 활용 계획

1. 열목어의 친어 관리/종묘 생산에 유용하게 사용될 수 있는 유전자 재조합 단백질의 혈액 내 잔존성을 방사면역측정법으로 조사할 예정임
2. 연어과어류의 성숙 개체에서 난모 세포 발달을 촉진시키는 호르몬의 영향을 생체 내 실험으로 확인할 계획임
3. 정원세포 및 정소의 생식 세포 발달에 미치는 호르몬의 영향을 시험관 및 생체 내 실험을 통하여 조사할 필요성이 있음
4. 연어과 이외의 다른 어종 (잉어과 어류, 뱀장어 등)에서 호르몬의 생물학적 활성을 조사하여 타어종의 종묘 생산에 활용될 수 있는지 조사할 계획임

VI. 기대 효과

1. 경제적 측면 (성과의 산업계 이전 및 산업화를 통한 기대 효과 등)
 - 가. 수산물의 남획으로 인한 멸종 위기에 있는 해양 생물의 번식을 보존 유지 개발함으로써 국내 수산업에 큰 이익을 가져다 줄 것임
 - 나. 국제적인 특허를 획득할 가능성이 크므로 다양한 방면의 국부를 창출할 수 있음
 - 다. 향후 발생되어지는 새로운 유전학적인 기초 지식을 축적할 수 있음
2. 학술적 측면 (성과의 타 산업 및 학문에 미치는 효과 등)
 - 가. 수산 생물의 유전학적인 연구 능력과 분석력이 증진됨
 - 나. 자연 자원 조성을 위한 과학적이고 합리적인 방법 개발
 - 다. 어종 고유의 호르몬과 동일한 물질을 대량 확보 가능
 - 라. 적절한 번식 시기에 투여하는 인공 번식 방법의 이용 가능

S U M M A R Y

Pituitary glycoprotein hormones play a major role in fish reproductive activities. FSH (follicle-stimulating hormone), LH (luteinizing hormone) and TSH (thyroid-stimulating hormone) are belong to a glycoprotein hormone family in the pituitary of teleost fishes and their molecules are comprised of common α and distinct β subunit. In salmonid species, FSH mediates vitellogenesis and spermatogenesis, and LH regulates oocyte maturation and spermiation. Although it was suggested that fish TSH regulates the rate of metabolism by stimulating the thyroid gland to produce and release thyroid hormones as higher vertebrates, information on biological aspects of TSH is still limited.

In the present study, we firstly cloned cDNAs of glycoprotein α , FSH β and LH β subunits in an endangered salmonid species, Manchurian trout (*Brachymystax lenok*) living in nature-conservation sites designated by the Ministry of Environment in Korea. Next, to improve our understanding of the reproduction in this species we attempted to produce single-chain recombinant FSH (r-mtFSH) and LH (r-mtLH) proteins in Chinese hamster ovary (CHO-K1) cells and baculovirus-silkworm larvae system. In a transient transfection of the FSH and LH constructs into CHO-K1 cells followed by Western blot analysis, the tethered FSH in cell lysates or LH secreted into a medium was detected from 48 to 72 h after transfection. In addition, hemolymph containing r-mtFSH or r-mtLH was collected from silkworm larvae and purified by an affinity column. The r-mtFSH and r-mtLH were detected by Western blot analysis. In an *in vitro* assay, r-mtLH but not r-mtFSH stimulated the release of testosterone from maturing rainbow trout oocytes. Using cDNAs encoding FSH and LH receptors of amago salmon, we observed increased cAMP concentration in r-mtFSH and r-mtLH treated COS-7 cells. In sexually early recrudescence rainbow trout, r-mtFSH but not r-mtLH accelerated oocyte development. Taken together, these results suggest that the bioactive recombinant hormones could be applied for the induction of gonadal development and artificial propagation of endangered fishes.

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제 1 장 서 론

1절. 연구의 목적

강원도 고유의 냉수성 어종의 멸종을 방지하고 번식시키기 위한 생명 공학적 연구 방법을 검토하기 위하여 냉수성 고유어종의 생식선 자극 호르몬 cDNA를 cloning하고 단백질을 대량 생산하고자함.

2절. 연구 개발의 기술·경제·사회적 필요성

최근 우리 어민들의 초미의 관심사는 WTO시대의 도래와 함께 신자유주의 무역 체제에서 어떻게 하면 생존할 수 있을까 하는 것이다. 북미와 유럽 특히 미국의 대규모화된 기업형 양식가들은 자국정부의 방조에 가까운 유전자 재조합 농산물(GMO)의 확대 생산에 힘입어 저가의 수산물을 안정적으로 수출할 길을 모색하고 있으며, 중국은 값싼 노동력과 풍부한 수산물을 배경으로 수출한 결과 우리 어민의 생계에 치명적인 타격을 주고 있는 실정이다. 따라서 국민의 식생활 기호에 맞는 순수한 고유 어종을 보존하고 번식시켜 양식 산업화 시키는 것은 WTO체제에서 우리 어민이 생존할 수 있는 길의 하나라고 판단된다. 또한 산업기반시설이 빈약한 강원도지역의 특성을 고려할 때 국내 양식 산업을 새로 주도할 수 있는 메카로서의 재도약이 기대됨.

3절. 연구 개발의 목표 및 내용

1. 연구 개발의 최종 목표: 생명 공학을 통한 강원도 특산 어종의 번식 제어.
2. 일차 년도 : 생식선자극호르몬 cDNA의 cloning.
3. 이차 년도 : 호르몬의 인위적 대량 생산.
4. 삼차 년도 : 호르몬의 활성을 생체 내에서 확인.

제 2 장 국내외 연구 개발 현황

1절. 과제 관련 국내외 동향

1. 산업 수요 측면

가. 수산물의 남획으로 인한 생산력의 감소와 더불어 강원도 고유의 냉수성 어종의 멸종을 방지하고 번식시키기 위한.

나. 넙치, 조피 불락 등을 중심으로 양식어종의 단일화 추세는 수년간 지속되어 가격의 급락을 초래하고 있으며, 이에 따른 품질의 저하로 인하여 국민의 수산물에 대한 기피 현상을 불러일으킬 가능성이 있음. 따라서 양식어종의 다양화가 시급히 추진되어야 할 필요성이 있음.

2. 연구 수행 측면

가. 생물의 고유한 형질을 보존하기 위한 노력 즉, 종의 다양성을 유지하려는 선진 외국 과학자들의 장고의 연구 성과들은 최근 지구환경의 급격한 피폐화를 의식하게 된 자국민의 관심을 끌며, 암 발생의 기작에 관한 연구에 뒤를 이어 가장 주목받는 생명과학의 한 분야로 급부상하고 있음.

나. 북미, 북유럽, 이스라엘 그리고 일본에서는 종래의 양식 방법을 초월하여 순수한 고유 어종을 보존하고 번식시키는 양식 산업화를 추진 개발함으로써 멸종 위기의 해양 생물체를 보존하려는 연구를 생물 공학적 연구 방법을 통하여 수행하고 있음.

2절. 연구 개발 추진 전략 및 방법

1. 어류의 번식조절은 환경적요인 및 생리적 요인이 통합적으로 작용하여 이루어지며, 뱀장어와 같이 현재까지도 그 메카니즘에 대해서 잘 알려지지 않은 어종도 있음.
2. 생리적인 요인 중에서 뇌-뇌하수체-생식소로 이어지는 내분비 기관에서 생산되는 생식선 관련 호르몬은 번식 조절에서 중추적인 역할을 하고 있음이 잘 알려져 있음.
3. 한편, 어류의 번식을 조절하는 메카니즘의 이해와 산업적인 응용은 사람을 중심으로 한 포유동물의 연구 성과를 대부분 수용하는 차원에서 이루어지고 있는 현실을 부정하기 힘들며, 또한 실제로 많은 부분에서 유사한 측면이 있음.
4. 그러나 사람의 태반성 생식선 자극 호르몬(hCG)과 같이 포유류 유래의 호르몬은 어류에서 생식선 발달의 자극 효과는 있으나 생식선의 최종 발달단계 또는 부화된 자어에 좋지 않은 영향을 주는 것으로 많은 연구자들이 보고하고 있음.
5. 따라서 번식시키고자 하는 어종 고유의 호르몬과 동일한 물질을 대량 확보하여 적절한 번식 시기에 투여하는 인공 번식 방법이 유효하다고 할 수 있음.
6. 지난 10여 년간 어류에서 생식선 자극 호르몬(GTH)의 단백질 또는 cDNA가 분리되거나 cloning되어 있으나, 산업적으로 유용한 어류는 몇 종에 지나지 않으며 또한 대량 정제와 활성을 가진 호르몬의 생산에는 기술적인 한계가 있었음.
7. 가장 큰 이유로는 GTH가 2개의 subunit으로 구성되어 있으며 당단백질이기 때문이다.

따라서 대장균이외의 진핵 세포에서 대량으로 생산할 경우 당이 수식되어 원래의 형태에 가까운 단백질이 될 가능성이 매우 크다. 따라서 CHO 세포주에서 호르몬의 생산을 유도하여 정제한 후 어류에 주사하는 방법이 유효하다고 사료됨.

제 3 장 연구 개발 수행 내용 및 결과

1절. 연구의 목적:

강원도 고유의 냉수성 어종의 멸종을 방지하고 번식시키기 위한 생명 공학적 연구 방법을 검토하기 위하여 냉수성 고유 어종의 생식선 자극 호르몬 cDNA를 cloning하고 단백질을 대량 생산하고자 한다.

2절. 연구 개발의 목표 및 내용:

연구 개발의 최종 목표: 생명 공학을 통한 강원도 특산 어종의 번식 제어.

1. 1차 년도 : 생식선 자극 호르몬 cDNA의 cloning.
2. 2차 년도 : 생식선 자극 호르몬의 유전 공학적 생산.
3. 3차 년도 : 생식선 자극 호르몬의 생물학적 활성 조사.

3절. 서론

경골어류의 뇌하수체에서는 당 단백질성 호르몬류가 생산되는데, 두 종류의 생식선 자극 호르몬 (GTHs: FSH와 LH)과 갑상선자극호르몬(TSH)이 당 단백질 패밀리에 속한다. 이들 호르몬들은 공통적인 α 쇄와 호르몬 특이적인 β 쇄를 가진다(Swanson and Dittman, 1997; Blazquez *et al.*, 1998).

연어과 어종들에서, 현재까지 FSH는 난황 형성과 정자 형성의 역할을 하며, LH는 배우자의 최종 성숙을 조절한다고 알려져 있다(Suzuki *et al.*, 1988a, 1988; Swanson, 1991, Swanson and Dittman, 1997; Blazquez *et al.*, 1998).

냉수성 고유 어종인 열목어의 멸종을 방지하고 번식시키기 위한 생명 공학적 연구 방법을 검토하기 위하여 GTH α , FSH β , LH β 쇄를 연구 제1차 년도에 cloning하여 염기서열을 결정하였으며, 제2차 년도에서는 이들 유전자를 활용하여 진핵 세포에서 유전공학적으로 생산할 수 있는 방법을 모색하였다. 제3차 년도에서는 GTH의 기능을 활성화 하고 subunit 간의 유연성을 높이고자 NCS(N-linked carbohydrate sequence) 영역을 β 와 α -subunit 사이에 첨가하여 생산된 호르몬의 생물학적 활성을 시험관 내 및 생체 내에서 조사하였다(Klein *et al.*, 2003).

4절. 생식선 자극 호르몬 cDNA의 cloning

1. Sample의 수집

본 연구에 이용한 열목어는 2002년 삼척 내수면 연구소로부터 수용된 것으로 성숙 초기의 암컷개체(평균체중: 190.6g)를 선별하여 뇌하수체를 적출한 뒤 액체 질소에 급속 냉동 한 후 -80°C 에서 mRNA 추출 전까지 보관하였다.

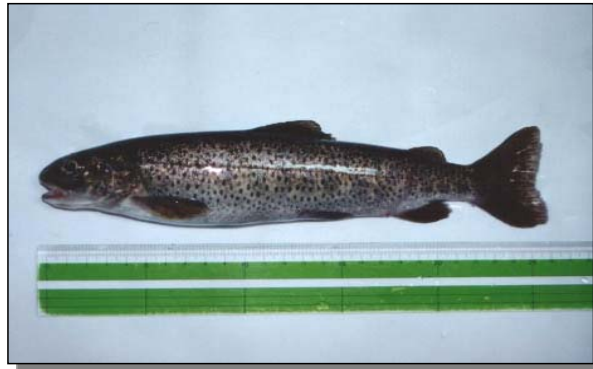


Fig. 1. Manchurian trout (*Brachymystax lenok*)

Classification : teleost, order salmon, fresh-water fish of family salmon

Distribution : Korea, Siberia, Europe etc.

2. 뇌하수체에서 mRNA 추출 및 cDNA 합성

열목어의 뇌하수체에서 mRNA를 추출 (QuickPrep™ Micro mRNA Purification Kit, Amersham Pharmacia biotech) 한 뒤 cDNA로 합성 (First-strand cDNA Synthesis Kit, Amersham Pharmacia biotech)하였다.

3. 호르몬 영역의 Oligo primer 제작 및 polymerase chain reaction (PCR)

산천어 GTH α , FSH β , LH β 각각의 염기 서열 (NCBI Accession number S69273, S69275, S69276)을 기초로 하여 oligo primer를 제작하였다. 뇌하수체에서 합성한 cDNA를 주형으로 사용하여 PCR 방법으로 GTH α , FSH β , LH β 를 각각 증폭하였다.

Table 1. Oligonucleotides used for sequence determination, PCR, and GTH α , FSH β , LH β

Primers	Directions	Sequence
GTH α -EcoR I	F	5'-GCGAATTCATGTGCTTGTTGAAATCAA-3'
GTH α -Xho I	R	5'-CGCTCGAGACAGTTTATGACTTATGG-3'
FSH β -EcoR I	F	5'-GCGAATTCATGTACTGCACCCACTTAAAG-3'
FSH β -Xho I	R	5'-CGCTCGAGTTACATTTCTAGTGGGTTTA-3'
LH β -EcoR I	F	5'-GCGAATTCATGTTAGGTCTTCATGTAGG-3'
LH β -Xho I	R	5'-CGCTCGAGTCACCACATGTCACCATC-3'

F, forward direction; R, reverse direction

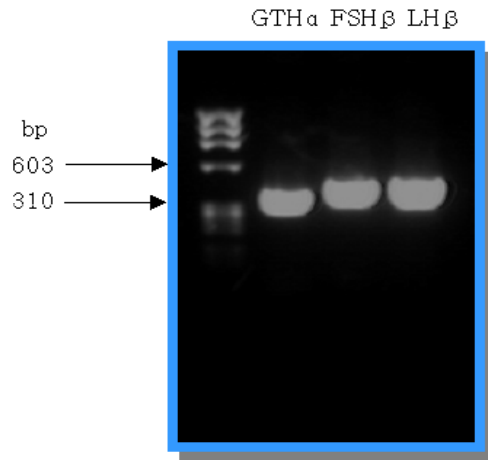


Fig. 2. PCR amplification for gonadotropin subunits

4. Cloning 및 plasmid DNA추출

각각의 증폭된 산물을 pcDNA3 (Invitrogen, USA) vector에 삽입하여 *E. coli* 형질전환 한 뒤 plasmid DNA를 추출하여 5', 3'-말단에 인식 부위로 붙여준 효소로 절단하여 insert의 유무를 확인한 다음 염기서열을 결정하였다.

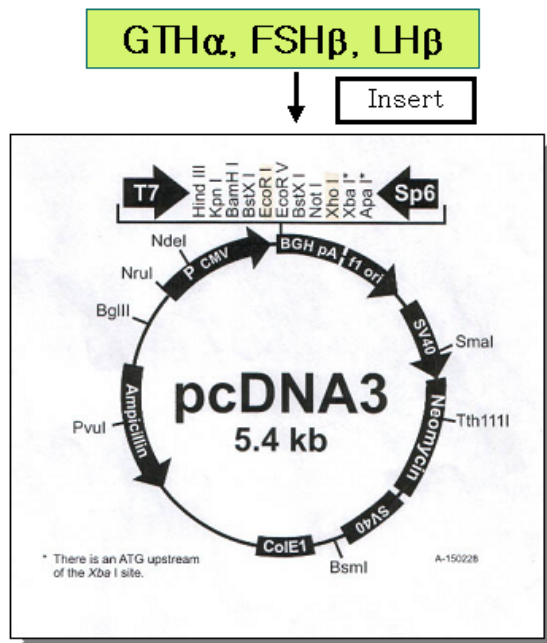


Fig. 3. pcDNA3 vector image

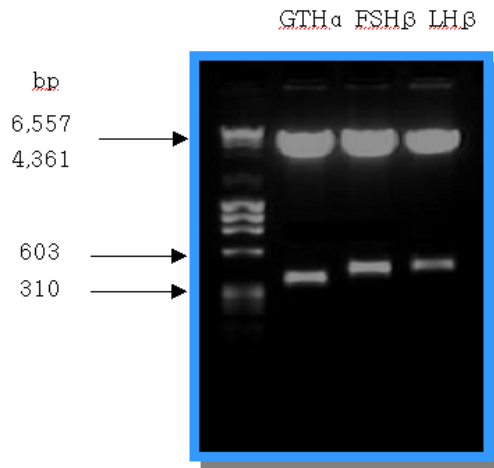
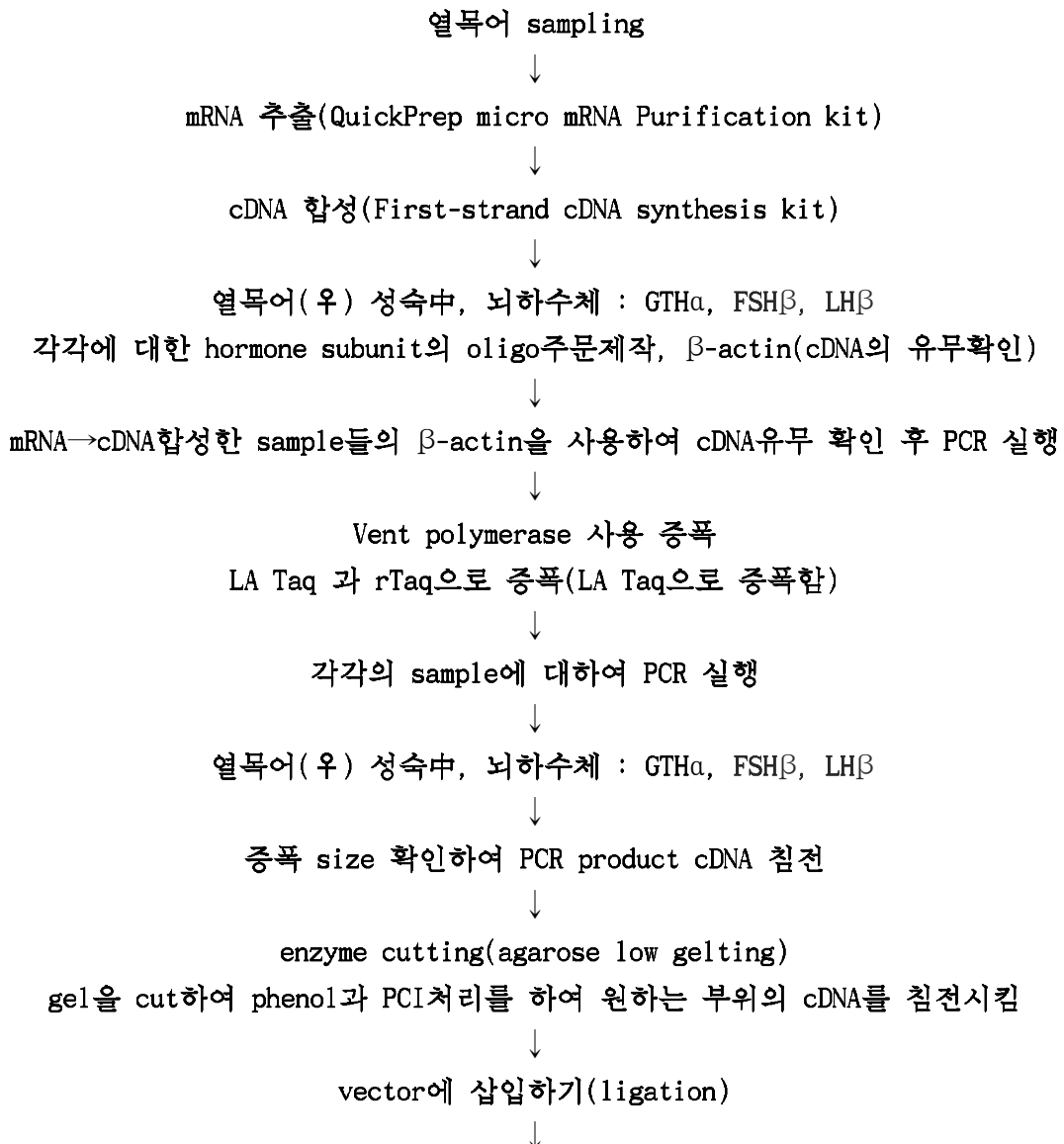
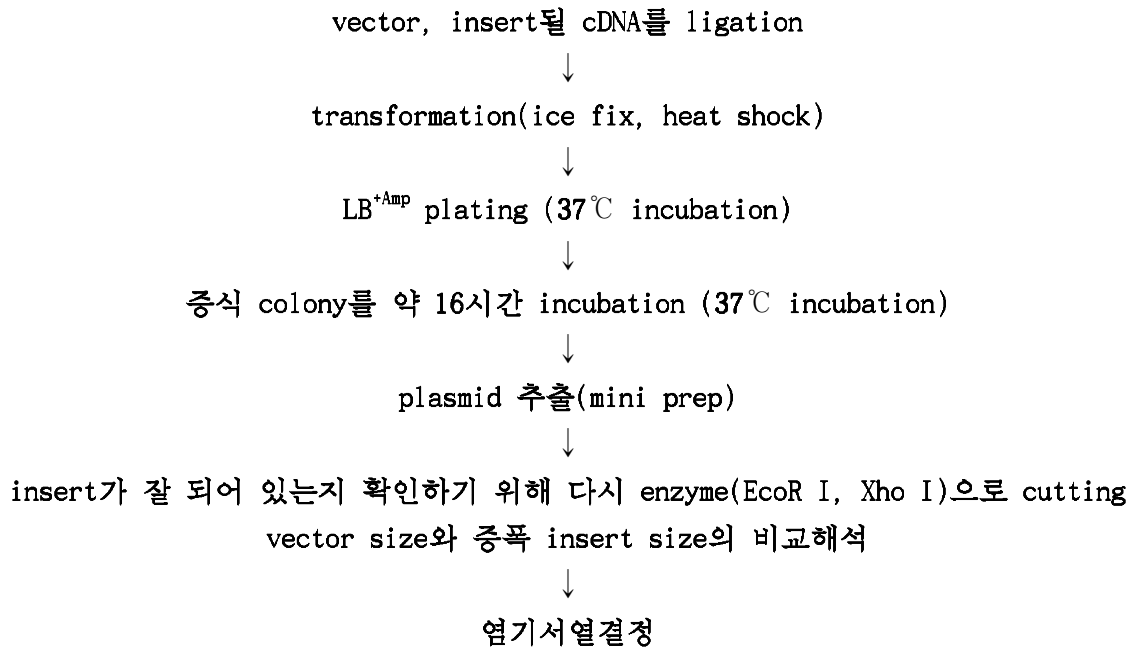


Fig. 4. Confirmation of inserted gonadotropin subunits

5. 실험 순서 요약도





6. Sequencing 결과

Sample : 열목어(우) 성숙中, 뇌하수체 : GTH α , FSH β , LH β

· 열목어는 GTH α , FSH β , LH β 의 sequence가 NCBI의 연어목 연어과인 산천어의 sequence와 비교했을 때 amino acid의 상동성은 각각 95%, 92%, 97% 보였다.

가. nucleotide 와 amino acid의 염기서열 해석결과

(1) 산천어 GTH α open reading frame

```

1 atgtgcttgttgaaatcaattggagtatccctcatcctgtccata
  M C L L K S I G V S L I L S I
46 cttctgtacatggcagactcttatccaaacagtgacatgacaaac
  L L Y M A D S Y P N S D M T N
91 gtgggctgtgaggaatgcaaaactgaaggagaacaaactattctca
  V G C E E C K L K E N K L F S
136 aaccggggcgcccctgtctaccagtgtacaggctgtgcttttcc
  N P G A P V Y Q C T G C C F S
181 agagcttatccaaccccactgcagtctaagaaggccacgttggtc
  R A Y P T P L Q S K K A T L V
226 ccaagaacatcacctctgaagccacatgctgcgttgcaaaagaa
  P K N I T S E A T C C V A K E
271 ggggaaagggtggtggtggacaacatcaagctgacgaaccacaca
  G E R V V V D N I K L T N H T
316 gagtgttggtgcaacacctgttatcaccataaatcataa 354
  E C W C N T C Y H H K S *

```


(2) 산천어 FSH β open reading frame

```
1 atgtactgcacccacttaaagacgctgcagctggctcgtcatggca
  M Y C T H L K T L Q L V V M A
46 acgctgtgggtgacaccagtgagggcggggacagactgcaggtat
  T L W V T P V R A G T D C R Y
91 ggctgccgactgaacaacatgaccatcaccgtggagagagaggac
  G C R L N N M T I T V E R E D
136 tgtcacggaagcatcaccatcaccacctgcgccggcctgtgcgaa
  C H G S I T I T T C A G L C E
181 acgacggacctgaactatcagagcacatggctgccacgctcccag
  T T D L N Y Q S T W L P R S Q
226 ggggtgtgtaacttcaaggagtggctcctacgagaaggtctacctg
  G V C N F K E W S Y E K V Y L
271 gaaggctgtccatccggggtcgacccttcttcataccctggcc
  E G C P S G V D P F F I P V A
316 aagagctgogattgcatcaaatgcaagacggacaacaccgactgt
  K S C D C I K C K T D N T D C
361 gatcgaaaggcatggcaacaccagctgcatcgtaaaccacta
  D R K G M A T P S C I V N P L
406 gaaatgtaa 414
  E M *
```

(3) 산천어 LH β open reading frame

```
1 atgtaggtcttcatgtaggcaccttgatctccctgtttctgtgc
  M L G L H V G T L I S L F L C
46 attctcctggaacccgttgaggggtctctcatgcagccctgtcag
  I L L E P V E G S L M Q P C Q
91 cccatcaaccagactgtgtctctggagaaggaaggctgcccaacg
  P I N Q T V S L E K E G C P T
136 tgcttagtcattcaaaccctatctgcagtggccactgcatcacc
  C L V I Q T P I C S G H C I T
181 aaggagccggttttcaagagcccattttccaccgtgtaccagcat
  K E P V F K S P F S T V Y Q H
226 gtgtgcacctatcgggacgtccgctatgaaatgatccgcctacct
  V C T Y R D V R Y E M I R L P
271 gactgtcccccttgggtggaccctcatgtcacctaccctgtggct
  D C P P W V D P H V T Y P V A
316 ctgagctgtgactgcagcctctgtaacatggacacttctgactgt
  L S C D C S L C N M D T S D C
361 accatcgagagcctgcagccagacttctgcattacccaaagagta
  T I E S L Q P D F C I T Q R V
406 ctaacggatggtgacatgtggtga 429
  L T D G D M W *
```

(4) 열목어 GTHa open reading frame

```
1 atgtgcttggtgaaatcaactggagtatccctcatcctgtccgta
  M C L L K S T G V S L I L S V
46 cttgtattcatcgcagactcttatccaaacagtgacaagacaaac
  L V F I A D S Y P N S D K T N
91 atgggctgtgaggaatgcacactgaagccgaacacaatcttccc
  M G C E E C T L K P N T I F P
136 aacatcatgcagtgtagcaggctgctgcttctccagagcttatcca
  N I M Q C T G C C F S R A Y P
181 acccactacggtccaagcaaaccatgctggtccccaagaacatc
  T P L R S K Q T M L V P K N I
226 acctctgaagccacgtgctgcgttgcaaaagaaggggaaagggtc
  T S E A T C C V A K E G E R V
271 accaccaaggatggcttcccggtagcgaaccacacagagtgtcac
  T T K D G F P V T N H T E C H
316 tgcagcacctggttattaccataaatcataa 345
  C S T C Y Y H K S *
```

(5) 열목어 FSH β open reading frame

```
1 atgtactgcaccacttaagacgctgcagctggtcatcatggca
  M Y C T H L K T L Q L V I M A
46 acgctgtggatgacaccagtgagggcagggacagactgctggtat
  T L W M T P V R A G T D C W Y
91 ggctgccgactaaacaacatgaccatcaccgtggagagagaggac
  G C R L N N M T I T V E R E D
136 tgtcacggaagcatcaccatcaccacctgcgccggcctgtgcgaa
  C H G S I T I T T C A G L C E
181 acgacggatctgaactaccagagcacatggatgccacgctcccag
  T T D L N Y Q S T W M P R S Q
226 gtggcgtgtaacttcaaggagtggctcctacgagaaggtctacctg
  V A C N F K E W S Y E K V Y L
271 gaaggctgtccatccggggccgacccttcttcttcttcttcttcttcc
  E G C P S G A D P F F I P V A
316 aagagctgogattgcatcaaatgcaagactgacaacaccgactgt
  K S C D C I K C K T D N T D C
361 gatcgcataagcatggcaacaccagctgcgtagtaaacccacta
  D R I S M A T P S C V V N P L
406 gaaatgtaa 414
  E M *
```

(6) 열독어 LHβ open reading frame

```
1 atgtaggtcttcatgtaggcaccttgatctccctgttcctgtgc
  M L G L H V G T L I S L F L C
46 atcctcctggaacccgttgaggggtctctcatgcagccctgtcag
  I L L E P V E G S L M Q P C Q
91 cccatcaaccagactgtgtctctggagaaggaaggctgccaacg
  P I N Q T V S L E K E G C P T
136 tgcttagtcattcaaacctatctgcagtggccactgcgtcacc
  C L V I Q T P I C S G H C V T
181 aaggagccggttttcaagagcccattttccaccgtgtaccagcat
  K E P V F K S P F S T V Y Q H
226 gtgtgcacctaccgagacgtccgctatgaaacgatccgcctacct
  V C T Y R D V R Y E T I R L P
271 gactgtcccccttgggtggaccctcatgtcacctaccctgtggct
  D C P P W V D P H V T Y P V A
316 ctgagctgtgactgcagcctctgtaacatggacacttctgactgt
  L S C D C S L C N M D T S D C
361 accatcgagagcctgcagccagacttctgcattacccaaagagta
  T I E S L Q P D F C I T Q R V
406 ctaacggatggtgacatgtggtga 429
  L T D G D M W *
```

나. 열목어와 NCBI의 등록된 산천어와의 nucleotide 및 amino acid sequence 비교

(1) nucleotide sequence 비교

(가) GTHa nucleotide

1) *Oncorhynchus masou*(=masu salmon) gonadotropin alpha

Score = 620 bits (313), Expect = 6e-175

Identities = 337/345 (97%), Gaps = 0/345 (0%)

Strand=Plus/Plus

Manchurian trout :1 ATGTGCTTGTTGAAATCAACTGGAGTATCOOCTCATOCTGTCCGTAAGTACTTGTATTTCATCGCA 60
|||||

Masu salmon :12 ATGTGCTTGTTGAAATCAACTGGACTATCOOCTCATCTTATCOGCACTTCTAGTCATCGCA 71

Manchurian trout : 61 GACTCTTATOC AAACAGTGACAAGACAAACATGGGCTGTGAGGAATGCACACTGAAGCOG 120
|||||

Masu salmon : 72 GACTCTTATOC AAACAGTGACAAGACAAACATGGGCTGTGAGGAATGCACACTGAAGCOG 131

Manchurian trout :121 AACACAATCTTCCCCAACATCATGCAGTGTACAGGCTGCTGCTTCTOCAGAGCTTATCCA 180
|||||

Masu salmon :132 AACACAATCTTCCCCAACATCATACAGTGTACAGGCTGCTGCTTCTOCAGAGCTTATCCA 191

Manchurian trout :181 ACCCCACTAOGGTCCAAGCAAACCATGCTGGTCCCCAAGAACATCAOCTCTGAAGCCACG 240
|||||

Masu salmon :192 ACCCCACTAOGGTCCAAGCAAACCATGCTGGTCCCCAAGAACATCAOCTCTGAAGCCACA 251

Manchurian trout :241 TGCTGCGTTGCAAAAGAAGGGGAAAGGGTCACCAACAAGGATGGCTTCCCAGGTGACGAAC 300
|||||

Masu salmon ; 252 TGCTGCGTTGCAAAAGAAGGGGAAAGGGTCACCAACAAGGATGGCTTCCCAGGTGACGAAC 311

Manchurian trout : 301 CACACAGAGTGTCACTGCAGCACCTGTTATTACCATAAATCATAA 345
|||||

Masu salmon : 312 CACACAGAGTGTCACTGCAGCACCTGTTATTACCATAAATCATAA 356

2) *Oncorhynchus mykiss*(=Rainbow trout) gonadotropin alpha

Score = 620 bits (313), Expect = 6e-175
Identities = 337/345 (97%), Gaps = 0/345 (0%)
Strand=Plus/Plus

Manchurian trout :1 ATGTGCTTGTTGAAATCAACTGGAGTATCCCTCATCCTGTCCGTA CTTGTATTCATOGCA 60
|||||
Rainbow trout :1 ATGTGCTTGTTGAAATCAACTGGACTATTCCTCATCCTGTCCGCAC TTTCTAGTCATOGCA 60

Manchurian trout :61 GACTCTTATCCAAACAGTGACAAGACAAACATGGGCTGTGAGGAATGCACACTGAAGCOG 120
|||||
Rainbow trout :61 GACTCTTATCCAAACAGTGACAAGACAAACATGGGCTGTGAGGAATGCACACTGAAGCOG 120

Manchurian trout:121 AACACAATCTTCCCCAACATCATGCAGTGTACAGGCTGCTGCTTCTOCAGAGCTTATCCA 180
|||||
Rainbow trout :121 AACACAATCTTCCCCAACATCATGCAGTGTACAGGCTGTTGCTTCTOCAGAGCTTATCCA 180

Manchurian trout:181 ACCCCACTAOGGTCCAAGCAAACCATGCTGGTCCCCAAGAACATCACCTCTGAAGCCACG 240
|||||
Rainbow trout :181 ACCCCACTAOGGTCCAAGCAAACCATGCTGGTCCCCAAGAACATCACCTCTGAAGCCACA 240

Manchurian trout :241 TGCTGOGTTGCAAAGAAGGGGAAAGGGTCACCACCAAGGATGGCTTCCCGGTGAOGAAC 300
|||||
Rainbow trout :241 TGCTGOGTTGCAAAGAAGGGGAAAGGGTCACCACCAAGGATGGCTTCCCGGTGAOGAAC 300

Manchurian trout :301 CACACAGAGTGTCACTGCAGCACCTGTTATTACCATAAATCATAA 345
|||||
Rainbow trout :301 CACACAGAGTGTCACTGCAGCACCTGCTATTACCATAAATCATAA 345

3) *Oncorhynchus kisutch*(=coho salmon) glycoprotein hormone alpha

Score = 603 bits (304), Expect = 1e-169
Identities = 328/336 (97%), Gaps = 0/336 (0%)
Strand=Plus/Plus

Manchurian trout :10 TTGAAATCAACTGGAGTATCOOCTCATOCTGTCCGTACTTGTATTGATOGCAGACTCTTAT 69
 |||||
 coho salmon :1 TTGAAATCAACTGGACTATCOOCTCATOCTGTCCGCACTTCTAGTCATCGGAGACTCTTAT 60

Manchurian trout:70 CCAAACAGTGACAAGACAAACATGGGCTGTGAGGAATGCACACTGAAGCOGAACACAATC 129
 |||||
 coho salmon :61 CCAAACAGTGACAAGACAAACATGGGCTGTGAGGAATGCACACTGAAGCOGAACACAATC 120

Manchurian trout :130 TTCCCCAACATCATGCAGTGTACAGGCTGCTGCTTCTOCAGAGCTTATCCAACCCACTA 189
 |||||
 coho salmon ; 121 TTCCCCAACATCATGCAGTGTACAGGCTGCTGCTTCTOCAGAGCTTATCCAACCCACTA 180

Manchurian trout :190 CGGTCCAAGCAAACCATGCTGGTCCCCAAGAACATCAOCTCTGAAGOCACGTGCTGOGTT 249
 |||||
 coho salmon : 181 CGGTCCAAGCAAACCATGCTGGTTCOCAAGAACATCAOCTCTGAAGOCACATGCTGTGTT 240

Manchurian trout :250 GC AAAAGAAGGGGAAAGGGTCAACCACCAAGGATGGCTTCCCGGTGACGAACCACACAGAG 309
 |||||
 coho salmon : 241 GC AAAAGAAGGGGAAAGGGTCAACCACCAAGGATGGCTTCCCGGTGACGAACCACACAGAG 300

Manchurian trout : 310 TGTCACTGCAGCACCTGTTATTACCATAAATCATAA 345
 |||||
 coho salmon : 301 TGTCACTGCAGCACCTGTTATTACCATAAATCATAA 336

4) *A. anguilla* (=European eel) glycoprotein hormone alpha

Score = 79.8 bits (40), Expect = 5e-12
 Identities = 79/92 (85%), Gaps = 0/92 (0%)
 Strand=Plus/Plus

Manchurian trout :157 TGCTGCTTCTOCAGAGCTTATCCAACCCACTACGGTCCAAGCAAACCATGCTGGTCCC 216
 |||||
A. anguilla :179 TGCTGTTTCTOCAGGGGGTACCCAACACCACTGCGGTCCAAGAAGACCATGCTGGTGOCA 238

Manchurian trout :217 AAGAACATCACCTCTGAAGCCACGTGCTGCGT 248
| | | | | | | | | | | | | | | | | | | | | | | |
A.anguilla ;239 AAGAACATCACATCTGAGGCAACGTGCTGCGT 270

Score = 48.1 bits (24), Expect = 0.017
Identities = 39/44 (88%), Gaps = 0/44 (0%)
Strand=Plus/Plus

Manchurian trout : 297 GAACCACACAGAGTGTCACTGCAGCACCTGTTATTACCATAAAT 340
| | | | | | | | | | | | | | | | | | | | | | | |
A.anguilla : 310 GAACCACACAGACTGCCACTGCAGCACCTGCTACTACCACAAAT 353

5) *Danio rerio*(= zebrafish) glycoprotein hormones, alpha polypeptide

Score = 73.8 bits (37), Expect = 3e-10
Identities = 88/105 (83%), Gaps = 0/105 (0%)
Strand=Plus/Plus

Manchurian trout : 154 GGCTGCTGCTTCTCCAGAGCTTATCCACCCCACTACGGTCCAAGCAAACCATGCTGGTC 213
| | | | | | | | | | | | | | | | | | | | | | | |
Danio rerio : 202 GGCTGCTGCTTTTCGAGAGCTTACCCACACCCCTGAGGTCCAAGAAAACCATGCTAGTC 261

Manchurian trout : 214 CCCAAGAACATCACCTCTGAAGCCACGTGCTGCGTTGCAAAAGAA 258
| | | | | | | | | | | | | | | | | | | | | | | |
Danio rerio : 262 CCAAAAAACATCACATCAGAAGCCACTTGCTGTGTAGCAAAAGAA 306

Score = 50.1 bits (25), Expect = 0.004
Identities = 37/41 (90%), Gaps = 0/41 (0%)
Strand=Plus/Plus

Manchurian trout : 298 AACCACACAGAGTGTCACTGCAGCACCTGTTATTACCATAA 338
| | | | | | | | | | | | | | | | | | | | | | | |
Danio rerio : 340 AACCACACAGACTGCCACTGCAGCACCTGTTACTATCATAA 380

(ㄴ) FSH β nucleotide

1) *Oncorhynchus masou*(=masu salmon) gonadotropin I beta

Score = 662 bits (334), Expect = 0.0

Identities = 394/414 (95%)

Strand = Plus / Plus

```
Manchurian trout: 1 atgtactgacccacttaaagacgctgcagctggatcatcatggcaacgctgtggatgaca 60
                    |||
Masu salmon : 51 atgtactgacccacttaaagacgctgcagctggatcatcatggcaacgctgtggatgaca 110

Manchurian trout: 61 ccagtgagggcagggacagactgctggatggctgccgactaacaacatgaccatcacc 120
                    |||
Masu salmon : 111 ccagtgagggcagggacagactgctggatggctgccgactgaacaacatgaccatcacc 170

Manchurian trout: 121 gtggagagagaggactgtcacggaagcatcaccatcaccacctgcgccggcctgtgcgaa 180
                    |||
Masu salmon : 171 gtggagagagaggactgtcacggaagcatcaccatcaccacctgcgccggcctgtgcgaa 230

Manchurian trout: 181 acgacggatctgaactaccagagcacatggatgccacgctcccaggtggcgtgtaacttc 240
                    |||
Masu salmon : 231 acgacggacctgaactatcagagcacatggatgccacgctcccagggggtgtaacttc 290

Manchurian trout: 241 aaggagtggctctacgagaaggcttacctggaaggctgtccatccggggccgacccttc 300
                    |||
Masu salmon : 291 aaggagtggctctacgagaaggcttacctggaaggctgtccatccgggggtcgagcccttc 350

Manchurian trout: 301 ttcattcctgttgccaagagctgcgattgcatcaaatgcaagactgacaacaccgactgt 360
                    |||
Masu salmon : 351 ttcataccggttgccaagagctgcgattgcatcaaatgcaagacggacaacaccgactgt 410

Manchurian trout: 361 gatcgcataagcatggcaacaccagctgtagtaaacaccactagaaatgtaa 414
                    |||
Masu salmon : 411 gatcgcaaaagtatggcaacaccagctgcatcgtaaaccactagaaatgtaa 464
```

2) *Oncorhynchus mykiss*(=Rainbow trout) gonadotropin I beta

Score = 681 bits (354), Expect = 0.0

Identities = 394/414 (95%)

Strand = Plus / Plus

Manchurian trout: 1 atgtactgcacccacttaaagacgctgcagctggcatcatggcaacgctgtggatgaca 60
|||||

Rainbow trout: 27 atgtactgcacccacttaaagatgctgcagctggcgtcatggcaacgctgtgggtgaca 86

Manchurian trout: 61 ccagtgagggcagggacagactgctggatggctgccgactaaacaacatgaccatcacc 120
|||||

Rainbow trout: 87 ccagtgagggcggggacagactgcaggtatggctgccgactaaacaacatgaccatcacc 146

Manchurian trout: 121 gtggagagagaggactgtcacggaagcatcaccatcaccacctgcgccggcctgtgcgaa 180
|||||

Rainbow trout: 147 gtggagagagaggactgtcacggaagcatcaccgtcaccacctgcgccggcctgtgcgaa 206

Manchurian trout: 181 acgacggatctgaactaccagagcacatggatgccacgctcccaggtggcgtgtacttc 240
|| |||||

Rainbow trout: 207 acaacggacctgaactatcagagcacatggctgccgcgctcccaggggtgtgtacttc 266

Manchurian trout: 241 aaggagtggctctacgagaaggcttacctggaaggctgtccatccggggccgacccttc 300
|||||

Rainbow trout: 267 aaggagtggctctacgagaaggcttacctggaaggctgtccatccgggggtcaacccttc 326

Manchurian trout: 301 ttattcctgttgccaagagctgcgattgcatcaaatgcaagactgacaacaccgactgt 360
|||||

Rainbow trout: 327 ttcataccgttgccaagagctgcgattgcatcaaatgcaagacggacaacaccgactgt 386

Manchurian trout: 361 gatcgcataagcatggcaacaccagctgcgtagtaaaccactagaaatgtaa 414
|||||

Rainbow trout: 387 gatcgcataagcatggcaacaccagctgcatagtaaaccactagaaatgtaa 440

3) *Oncorhynchus kisutch*(=Coho salmon) follicle stimulating hormone beta

Score = 686 bits (346), Expect = 0.0

Identities = 391/406 (96%), Gaps = 0/406 (0%)

Strand=Plus/Plus

Manchurian trout : 9 CACCCACTTAAAGACGCTGCAGCTGGTCATCATGGCAAAGCTGTGGATGACACCAGTGAG 68
|||||

Coho salmon : 1 CACCCACTTAAAGACGCTGCAGCTGGTCATCATGGCAAAGCTGTGGGTGACACCAGTGAG 60

Manchurian trout : 69 GGCAGGGACAGACTGCTGGTATGGCTGCOGACTAAACAACATGAOCATCACCGTGGAGAG 128
||| |||

Coho salmon : 61 GGCGGGGACAGACTGCAGGTATGGCTGCOGACTAAACAACATGAOCATCACCGTGGAGAG 120

Manchurian trout : 129 AGAGGACTGTCAOCCAAGCATCACCATCAOCCACTGCGOCCGCTGTGOGAAAACGACGGA 188
|||||

Coho salmon : 121 AGAGGACTGTCAOCCAAGCATCACCATCAOCCACTGCGOCCGCTGTGOGAAAACGACGGA 180

Manchurian trout : 189 TCTGAACTACCAGAGCACATGGATGCCAAGCTOCCAGGTGGOGTGTAACTTCAAGGAGTG 248
|||||

Coho salmon : 181 OCTGAACTATCAGAGCACATGGCTGCCAAGCTOCCAGGGGGTGTGTAACTTCAAGGAGTG 240

Manchurian trout : 249 GTOCTAOGAGAAGGTCTAOCCTGGAAGGCTGTGCATCOGGGGGCOGACCCCTTCTTCATTOC 308
|||||

Coho salmon : 241 GTOCTAOGAGAAGGTCTAOCCTGGAAGGCTGTGCATCOGGGGGTOGACCCCTTCTTCATAOC 300

Manchurian trout : 309 TGTTGCCAAGAGCTGOGATTGCATCAAATGCAAGACTGACAACAACCGACTGTGATOGCAT 368
|||||

Coho salmon : 301 OGTGCCAAGAGCTGOGATTGCATCAAATGCAAGACGGACAACAACCGACTGTGATOGCAT 360

Manchurian trout : 369 AAGCATGGCAACACCCAGCTGCGTAGTAAACCCACTAGAAATGTAA 414
|||||

Coho salmon : 361 AAGCATGGCAACACCCAGCTGCATTGTAAACCCACTAGAAATGTAA 406

4) *Coregonus autumnalis* (=Baikal omul) gonadotropin I beta subunit

Score = 646 bits (326), Expect = 0.0

Identities = 392/414 (94%), Gaps = 0/414 (0%)

Strand=Plus/Plus

Manchurian trout : 1 ATGTA CTGCA CCCC ACTTAAAGACGCTGCAGCTGGTCATCATGGCAACGCTGTGGATGACA 60
|||||
omul : 64 ATGTA CTGCA CCCC ACTTAAAGGATGCTGCAGCTGGTGTGTCATGGCAACACTGTGGGTGACA 123

Manchurian trout : 61 CCAGTGAGGGGCAGGGACAGACTGCTGGTATGGCTGCOGACTAAACAACATGACCATCACC 120
|||||
omul : 124 CCAGTGAGGGGOGGGGACACACTGCAGGTATGGCTGCOGACTAAACAACATGACCATCACC 183

Manchurian trout : 121 GTGGAGAGAGAGGACTGTCAO GGAAGCATCACCATCA CCACTGOGCOGGCOCTGTGOGAA 180
|||||
omul : 184 GTGGAGAGAGAGGACTGTCAO GGAAGCATCACCATCA CCACTGOGCOGGCOCTGTGOGAA 243

Manchurian trout : 181 ACGACGGATCTGAACTACCAGAGCACATGGATGCCACGCTCCCAGGTGGCGTGTA ACTTC 240
||
omul : 244 ACAACGGATCTGAACTATCAGAGCACATGGCTGCCCTCGCTCCCAGGGGGCGTGTA ACTTC 303

Manchurian trout : 241 AAGGAGTGGTOCTACGAGAAGGTCTA OCTGGAAGGCTGTCCATCOGGGGGCOGA CCCCCTTC 300
|||||
omul : 304 AAGGAGTGGTOCTACGAGGAGGTCTA OCTGGAAGGCTGTCCACCCGGGGCOGA CCCCCTTC 363

Manchurian trout : 301 TTCATTCTGTTGCCAAGAGCTGOGATTGCATCAAATGCAAGACTGACAACACCGACTGT 360
|||||
omul : 364 TTCATACTGTTGCCAAGAGCTGTGATTGCATCAAATGCAAGACGGACAACACTGATTGT 423

Manchurian trout : 361 GATCGCATAAGCATGGCAACACCCAGCTGCGTAGTAAACCCACTAGAAATGTAA 414
|||||
omul : 424 GATCGCATAAGCATGGCAACACCCAGCTGTGTAGTAAACCCACTAGAAATGTAA 477

5) *Salmo salar* (=Atlantic salmon) gonadotropin I beta

Score = 589 bits (297), Expect = 2e-165

Identities = 351/369 (95%), Gaps = 0/369 (0%)

Strand=Plus/Plus

Manchurian trout : 38 TCATGGCAACGCTGTGGATGACACCAGTGAGGGCAGGGACAGACTGCTGGTATGGCTGOC 97
||||||| ||||| ||||||||| ||||||||| |||||||||

Atlantic salmon : 2 TCATGGCAATGCTGTGGGTGACACCAGTGAGGGCGGGGACAGACTGCAGGTATGGCTGOC 61

Manchurian trout : 98 GACTAAACAACATGACCATCAOOGTGGAGAGAGAGGACTGTCAOOGGAAGCATCAOCATCA 157
||||||| ||||||||| ||||||||| ||||||||| |||||||||

Atlantic salmon : 62 GACTAAACAGCATGACCATCAOOGTGGAGAGAGAGGACTGTCAOOGGAAGCATCAOCATCA 121

Manchurian trout : 158 CCAOCTGOGCOGGCOCTGTGOGAAACGAOOGATCTGAACTACCAGAGCACATGGATGOCAC 217
||||||| ||||||||| ||||||||| ||||||||| |||||

Atlantic salmon : 122 CCAOCTGOGCOGGCOCTGTGOGAAACGAOOGATATGAACTATCAGAGCACATGGCTGOCGC 181

Manchurian trout : 218 GCTOCCAGGTGGOGTGTAACTTCAAGGAGTGGTOCTACGAGAAGGTCTACCTGGAAGGCT 277
||||||| ||||||||| ||||||||| ||||||||| |||||||||

Atlantic salmon : 182 GCTOCCAGGGGGOGTGTAACTTCAAGGACTGGTOCTACGAGAAGGTCTACCTGGAAGGCT 241

Manchurian trout : 278 GTOCATCOGGGGGGOOGAOCOCCTTCTTCATTCTGTTGCOAAGAGCTGOGATTGCATCAAAT 337
||||||| ||||||||| || ||||||||| |||||||||

Atlantic salmon : 242 GTOCATCOGGGGGTTGACOCOCCTTCTTCATAOOCGTTGCOAAGAGCTGOGATTGCATCAAAT 301

Manchurian trout : 338 GCAAGACTGACAACACOGACTGTGATCGCATAAGCATGGCAACACOCAGCTGCGTAGTAA 397
|| ||||| ||||||||| ||||||||| ||||||||| |||||

Atlantic salmon : 302 GCGAGACGGACAACACOGACTGTGATCGCATAAGCATGGCAACACOCAGCTGCATAGTAA 361

Manchurian trout : 398 ACCCACTAG 406
|||||||

Atlantic salmon : 362 ACCCACTAG 370

(ㄷ) LHβ nucleotide

1) *Oncorhynchus masou*(=masu salmon) gonadotropin II beta

Score = 787 bits (397), Expect = 0.0

Identities = 421/429 (98%)

Strand = Plus / Plus

```
Manchurian trout: 1  atgtaggtcttcatgtaggcaccttgatctccctgttctctgtgcatcctcctggaacc 60
                      |||
Masu salmon   : 44  atgtaggtcttcatgtaggcaccttgatctccctgtctctgtgcattctcctggaacc 103

Manchurian trout: 61  gttgaggggtctctcatgcagccctgtcagcccatcaaccagactgtgtctctggagaag 120
                      |||
Masu salmon   : 104 gttgaggggtctctcatgcagccctgtcagcccatcaaccagactgtgtctctggagaag 163

Manchurian trout: 121 gaaggctgccaacgtgcttagtcattcaaacccctatctgcagtggccactgcgtcacc 180
                      |||
Masu salmon   : 164 gaaggctgccaacgtgcttagtcattcaaacccctatctgcagtggccactgcatcacc 223

Manchurian trout: 181 aaggagccggtttcaagagcccattttccaccgtgtaccagcatgtgtgcacctaccga 240
                      |||
Masu salmon   : 224 aaggagccggtttcaggagcccattttccaccgtgtaccagcatgtgtgcacctatcgg 283

Manchurian trout: 241 gacgtccgctatgaaacgatccgcctacctgactgtccccctgggtggaccctcatgtc 300
                      |||
Masu salmon   : 284 gacgtccgctatgaaatgatccgcctacctgactgtccccctgggtggaccctcatgtc 343

Manchurian trout: 301 acctaccctgtggctctgagctgtgactgcagcctctgtaacatggacacttctgactgt 360
                      |||
Masu salmon   : 344 acctaccctgtggctctgagctgtgactgcagcctctgtaacatggacacttctgactgt 403
```

Manchurian trout: 361 accatcgagagcctgcagccagacttctgcattacccaaagagtactaacggatggtgac 420
|||||

Masu salmon : 404 accatcgagagcctgcagccagacttctgcattacccaaagagtactaacggatggtgac 463

Manchurian trout: 421 atgtggtga 429
|||||

Masu salmon : 464 atgtggtga 472

2) *Oncorhynchus mykiss*(=Rainbow trout) gonadotropin II beta

Score = 790 bits (411), Expect = 0.0

Identities = 423/429 (98%)

Strand = Plus / Plus

Manchurian trout: 1 atgttaggtcttcatgtaggcaccttgatctccctgttctctgtgcatcctcctggaaccc 60
|||||

Rainbow trout: 34 atgttaggtcttcatgtaggcaccttgatctccctgttctctgtgcatcctcctggaaccc 93

Manchurian trout: 61 gttgaggggtctctcatgcagccctgtcagcccatcaaccagactgtgtctctggagaag 120
|||||

Rainbow trout: 94 gttgaggggtctctcatgcagccctgtcagcccatcaaccagactgtgtctctggagaag 153

Manchurian trout: 121 gaaggctgcccaacgtgcttagtcattcaaacccctatctgcagtggccactgcgtcacc 180
|||||

Rainbow trout: 154 gaaggctgcccaacgtgcttagtcattcaaacccctatctgcagtggccactgcgtcacc 213

Manchurian trout: 181 aaggagccggtttcaagagcccattttccaccgtgtaccagcatgtgtgcacctaccga 240
|||||

Rainbow trout: 214 aaggagccggtttcaagagcccattttccaccgtgtaccagcatgtgtgcacctaccgg 273

Manchurian trout: 241 gacgtccgctatgaaacgatccgcctacctgactgtcccccttgggtggaccctcatgtc 300
|||||

Rainbow trout: 274 gacgtccgctatgaaacgatccgcctacctgactgtcccccttgggtggaccctcatgtc 333

Manchurian trout: 301 acctaccctgtggctctgagctgtgactgcagcctctgtaacatggacacttctgactgt 360
|||||

Rainbow trout: 334 acctaccctgtggctctgagctgtgactgcagcctctgtaacatggacacttctgactgt 393

Manchurian trout: 361 accatcgagagcctgcagccagacttctgcattacccaagagtactaacggatggtgac 420
|||||

Rainbow trout: 394 accatcgagagcctgcagccagacttctgcattacgcaagagtactaacggatggtgac 453

Manchurian trout: 421 atgtggtga 429

|||||

Rainbow trout: 454 atgtggtga 462

3) *Onchorhynchus keta*(= Chum salmon) gonadotropin-II beta subunit

Score = 819 bits (413), Expect = 0.0

Identities = 425/429 (99%), Gaps = 0/429 (0%)

Strand=Plus/Plus

Manchurian trout : 1 ATGTTAGGICTTCATGTAGGCACCTTGATCTCCCTGTTCCCTGTGCATCCTCCTGGAACCC 60
|||||

Chum salmon : 56 ATGTTAGGICTTCATGTAGGCACCTTGATCTCCCTGTTCCCTGTGCATCCTCCTGGAACCC 115

Manchurian trout: 61 GTTGAGGGGICTCTCATGCAGCCCTGTCAGCCCATCAACCAGACTGTGTCTCTGGAGAAG 120
|||||

Chum salmon :116 GTTGAGGGGICTCTCATGCAGCCCTGTCAGCCCATCAACCAGACTGTGTCTCTGGAGAAG 175

Manchurian trout :121 GAAGGCTGCCCAACGTGCTTAGTTCATTCAAACCCCTATCTGCAGTGGCCACTGCGTCACC 180
|||||

Chum salmon :176 GAAGGCTGCCCAACGTGCTTAGTTCATTCAAACCCCTATCTGCAGTGGCCACTGCGTCACC 235

Manchurian trout :181 AAGGAGCCGGTTTTCAAGAGCCCATTTTCCACCGTGTACCAGCATGTGTGCACCTACCGA 240
|||||

Chum salmon :236 AAGGAGCCGGTTTTCAAGAGCCCATTTTCCACCGTGTACCAGCATGTGTGCACCTACCGG 295

Manchurian trout : 241 GACGTCGGCTATGAAACGATCCGCTACCTGACTGTCCCCCTTGGGTGGACCCCTCATGTC 300
|||||

Chum salmon : 296 GACGTCGGCTATGAAACGATCCGCTACCTGACTGTCCCCCTTGGGTGGACCCCTCATGTC 355

Manchurian trout : 301 ACCTACCCCTGTGGCTCTGAGCTGTGACTGCAGCCTCTGTAAACATGGACACTTCTGACTGT 360
|||||

Chum salmon : 356 ACCTACCCCTGTGGCTCTGAGCTGTGACTGCAGCCTCTGTAAACATGGACACTTCTGACTGT 415

Manchurian trout : 361 ACCATCGAGAGCCTGCAGCCAGACTTCTGCATTACCCAAAGAGTACTAACGGATGGTGAC 420
|||||

Chum salmon : 416 ACCATCGAGAGCCTGCAGCCAGACTTCTGCATTACCCAAAGAGTACTAACAGATGGTGAC 475

Manchurian trout : 421 ATGTGGTGA 429
|||||

Chum salmon : 476 ATGTGGTGA 484

4) *Oncorhynchus kisutch*(=coho salmon) luteinizing hormone beta

Score = 775 bits (391), Expect = 0.0

Identities = 409/415 (98%), Gaps = 0/415 (0%)

Strand=Plus/Plus

Manchurian trout : 15 TGIAGGCACCTTGATCTCCCTGTTCTGTGCATCCTCCTGGAACCCGTTGAGGGGTTCTCT 74
|||||

coho salmon : 1 TGIAGGCACCTTGATCTCCCTGTTCTGTGCATCCTCCTGGAACCCATGAGGGGTTCTCT 60

Manchurian trout : 75 CATGCAGCCCTGTGAGCCCATCAACCAGACTGTGTCTCTGGAGAAGGAAGGCTGCCCAAC 134
|||||

coho salmon : 61 CATGCAGCCCTGTGAGCCCATCAACCAGACTGTGTCTCTGGAGAAGGAAGGCTGCCCAAC 120

Manchurian trout : 135 GTGCTTAGTCATTCAAACCCCTATCTGCAGTGGCCACTGCGTCACCAAGGAGCCGGTTTT 194
|||||

coho salmon : 121 GTGCTTAGTCATTCAAACCCCTATCTGCAGTGGCCACTGCGTCACCAAGGAGCCGGTTTT 180

Manchurian trout : 195 CAAGAGCCCATTTTCCACCGTGTACCAGCATGTGTGCACCTACCGAGACGTCCGCTATGA 254
 |||
 coho salmon : 181 CAAGAGCCCATTTTCCACCGTGTACCAGCATGTGTGCACCTACCGGGACGTCCGCTATGA 240

Manchurian trout : 255 AACGATCCGCCTACCTGACTGTCCCCCTTGGGTGGACCCCTCATGTACCTACCCCTGTGGC 314
 |||
 coho salmon : 241 AACGATCCGCCTACCTGACTGTCCCCCGTGGGTGGACCCCTCATGTACCTACCCCTGTGGC 300

Manchurian trout : 315 TCTGAGCTGTGACTGCAGCCTCTGTAAACATGGACACTTCTGACTGTACCATCGAGAGCCT 374
 |||
 coho salmon : 301 TCTGAGCTGTGACTGCAGCCTCTGTAAACATGGACACTTCTGACTGTACCATCGAGAGCCT 360

Manchurian trout : 375 GCAGCCAGACTTCTGCATTACCCAAAGAGTACTAACGGATGGTGACATGTGGTGA 429
 |||
 coho salmon : 361 GCAGCCAGACTTCTGCATTACCCAAAGAGTACTAACGGATGGTGACATGTGGTGA 415

5) *Coregonus autumnalis* (=Baikal omul) gonadotropin II beta

Score = 676 bits (341), Expect = 0.0
 Identities = 400/419 (95%), Gaps = 3/419 (0%)
 Strand=Plus/Plus

Manchurian trout :1 ATGTTAGGICTTCATGTAGGCACCTTGAT—CTCCCTGTTCCCTGTGCATCCTCCTGGAA 57
 |||
 omul : 26 ATGTTAGGICTTCATGTAGGCACCTTGATGATCTCCCTGTTCCCTGTGCATCCTCCTGGAA 85

Manchurian trout : 58 CCCGTTGAGGGGTCCTCATGCAGCCCTGTTCAGCCCATCAACCAGACTGTGTCTCTGGAG 117
 ||
 omul : 86 CCTGTTGAGGGGTCCTCATGCAGCCCTGTTCAGCCCATCAACCAGACTGTGTCTCTGGAG 145

Manchurian trout :118 AAGGAAGGCTGCCCAACGTTGCTTAGTCAATTCAAACCCCTATCTGCAGTGGCCACTGCGTIC 177
 |||
 omul : 146 AAGGAAGGCTGCCCAACGTTGCTTAGTCAATTCAAACCCCATCTGCAGTGGCCACTGCTTC 205

Manchurian trout : 178 ACCAAGGAGCCCGTTTTCAAGAGCCCATTTTCCACCGTGTACCAGCATGTGTGCACCTAC 237
 |||
 omul : 206 ACCAAGGAGCTGGTTTTCAAGAGCCCATTTTCCACTGTGTACCAGCACGTGTGCACCTAC 265

Manchurian trout : 238 CGAGACGTCCGCTATGAAACGATCCGCTACCTGACTGTCCCCCTTGGGTGGACCCCTCAT 297
 |
 omul : 266 AGGGACGTCCGCTATGAAACGATCTGCCTACCTGACTGTTCCCCTTGGGTGGACCCCTCAT 325

Manchurian trout :298 GTCACCTACCTGTGGCTCTGAGCTGTGACTGCAGCCTCTGTAAACATGGACACTTCTGAC 357
 |||
 omul : 326 GTCACCTACCTGTGGCTCTGAGCTGTGACTGCAGCCTCTGTAAACATGGACACTTCCGAC 385

Manchurian trout : 358 TGTACCATCGAGAGCCTGCAGCCAGACTTCTGCATTACCCAAAGAGTACTAACGGATGG 416
 |||
 omul : 386 TGTACCATTGAGAGCCTGCAGCCAGACCTCTGCATGACTCAAAGAGTACTAGCGGATGG 444

다. amino acid sequence 비교

(1) GTHa amino acid

1) *Oncorhynchus masou*(=Masu salmon) gonadotropin alpha 2

Score = 238 bits (608), Expect = 1e-62
 Identities = 109/114 (95%), Positives = 112/114 (98%)
 Frame = +1

Manchurian trout: 1 MCLKKSTGVSLILSVLVFIADSYPNSDKTNMGCEECTLKPNTIFPNIMQCTGCCFSRAYP 180
 MCLKKSTG+SLILS L+ IADSYPNSDKTNMGCEECTLKPNTIFPNI+QCTGCCFSRAYP

Masu salmon : 1 MCLKKSTGLSLILSALLVIADSYPNSDKTNMGCEECTLKPNTIFPNIQCTGCCFSRAYP 60

Manchurian trout: 181 TPLRSKQTMLVPKNITSEATCCVAKEGERVTTKDGFPVTNHTECHCSTCYHKS 342
 TPLRSKQTMLVPKNITSEATCCVAKEGERVTTKDGFPVTNHTECHCSTCYHKS

Masu salmon : 61 TPLRSKQTMLVPKNITSEATCCVAKEGERVTTKDGFPVTNHTECHCSTCYHKS 114

2) *Oncorhynchus mykiss*(=Rainbow trout) gonadotropin alpha

Score = 238 bits (606), Expect = 5e-62

Identities = 109/114 (95%), Positives = 111/114 (96%)

Manchurian trout: 1 MCLLKSTGVSLILSVLFIADSYPNSDKTNMGCEECTLKPNITIFPNIMQCTGCCFSRAYP 60
MCLLKSTG+ LILS L+ IADSYPNSDKTNMGCEECTLKPNITIFPNIMQCTGCCFSRAYP
Rainbow trout: 1 MCLLKSTGLFLILSALLVIADSYPNSDKTNMGCEECTLKPNITIFPNIMQCTGCCFSRAYP 60

Manchurian trout: 61 TPLRSKQTMLVPKNITSEATCCVAKEGERVTTKDGFPVTNHTECHCSTCYHHKS 114
TPLRSKQTMLVPKNITSEATCCVAKEGERVTTKDGFPVTNHTECHCSTCYHHKS
Rainbow trout: 61 TPLRSKQTMLVPKNITSEATCCVAKEGERVTTKDGFPVTNHTECHCSTCYHHKS 114

3) *Hippoglossus hippoglossus*(=Atlantic halibut) gonadotropin alpha

Score = 144 bits (362), Expect = 1e-33

Identities = 69/114 (60%), Positives = 82/114 (71%), Gaps = 5/114 (4%)

Manchurian trout: 4 LKSTGVSLIL-SVLFIADSYPNSDKTNMGCEECTLKPNITIFPN---IMQCTGCCFSRAY 59
++S G+SLIL S ++IADSYPN++ +NMGCEECTL+ N F I QC GCCFS+AY
Atlantic halibut: 19 VRSAGLSLILLSFFLYIADSYPNTELSNMGCEECTLRNIFFFSTDRPIFQCVGCCFSQAY 78

Manchurian trout: 60 PTPLRSKQTMLVPKNITSEATCCVAKEGERVTTKDGFPVTNHTECHCSTCYHHK 113
PTPL++ TM PKNITSEATCCVAK T V NHTECHC TCY+HK
Atlantic halibut: 79 PTPLKAMNTMATPKNITSEATCCVAKHSYE-TEVAAIKVRNHTECHCHTCYHHK 131

4) *Mylopharyngodon piceus*(=Black carp) gonadotropin alpha

Score = 148 bits (373), Expect = 6e-35

Identities = 69/108 (63%), Positives = 79/108 (72%), Gaps = 5/108 (4%)

Manchurian trout: 11 LILSVLFIADSYPNSDKTNMGCEECTLKPNITIFPN----IMQCTGCCFSRAYPTPLRSK 66
L+ +L+ + YP +D N GCEEC LK N IF + QC GCCFSRAYPTPLRSK
Carp: 12 LFFMLIRLGQLYPRNDMNNFGCEECKLKENNIFSKPGAPVYQCMGCCFSRAYPTPLRSK 71

Manchurian trout: 67 QTMLVPKNITSEATCCVAKEGERVTTKDGFPVTNHTECHCSTCYHHKS 114
+TMLVPKNITSEATCCVAKE +RV D + NHT+CHCSTCYHHKS
Carp: 72 KTMLVPKNITSEATCCVAKEVKRVLVND-VKLVNHTDCHCSTCYHHKS 118

5) *Dicentrarchus labrax*(=sea bass) gonadotropin alpha

Score = 146 bits (368), Expect = 2e-34

Identities = 64/106 (60%), Positives = 81/106 (76%), Gaps = 4/106 (3%)

Manchurian trout: 11 LILSVLVFIADSYPSNSDKTNMGCEECTLKPNITIFPN---IMQCTGCCFSRAYPTPLRSKQ 67
L+LS ++++ DSYP+ D +NMGCEECTL+ N++F + QC GCCFSRAYPTPL++ +
sea bass: 12 LLLSFILYWDSYPSMDLSNMGCEECTLRKNSVFSRDRPVYQCMGCCFSRAYPTPLKAMK 71

Manchurian trout: 68 TMLVPKNITSEATCCVAKEGERVTTKDGFPVTNHTTECHCSTCYHK 113
TM +PKNITSEATCCVAK T G V NHT+CHCSTCY+HK
sea bass: 72 TMTIPKNITSEATCCVAKHSYE-TEVAGIKVRNHTDCHCSTCYFHK 116

(2) FSH β amino acid

1) *Oncorhynchus masou*(=Masu salmon) gonadotropin I beta

Score = 284 bits (726), Expect = 2e-76

Identities = 127/137 (92%), Positives = 132/137 (96%)

Frame = +1

Manchurian trout: 1 MYCTHLKTLQLVIMATLWMTVPV RAGTDCWYGCRLNMTITVEREDCHGSITITTCAGLCE 180
MYCTHLKTLQLV+MATLW+TPV RAGTDC YGCRLNMTITVEREDCHGSITITTCAGLCE
Masu salmon : 1 MYCTHLKTLQLW/MATLWTPV RAGTDCRYGCRLNMTITVEREDCHGSITITTCAGLCE 60

Manchurian trout: 181 TTDLNYQSTWMPRSQVACNFKEWSYEK WYLEGCPSGADPFFIPVAKSCDCIKCKTDNTDC 360
TTDLNYQSTW+PRSQ CNFKEWSYEK WYLEGCPSG +PFFIPVAKSCDCIKCKTDNTDC
Masu salmon : 61 TTDLNYQSTWLPRSQGVACNFKEWSYEK WYLEGCPSGVEPFFIPVAKSCDCIKCKTDNTDC 120

Manchurian trout: 361 DRISMATPSCVNVNPLEM 411
DR SMATPSC+VNVNPLEM
Masu salmon : 121 DRKSMATPSCIVNPLEM 137

2) *Oncorhynchus mykiss*(=Rainbow trout) gonadotropin I beta

Score = 281 bits (719), Expect = 4e-75

Identities = 125/137 (91%), Positives = 131/137 (95%)

Manchurian trout: 1 MYCTHLKTLQLVIMATLWMTTPVRAGTDCWYGCRLNNMTITVEREDCHGSITITTCAGLCE 60
MYCTHLK LQLV+MATLW+TPVRAGTDC YGCRLNNMTITVEREDCHGSIT+TTCAGLCE

Rainbow trout: 1 MYCTHLKMLQLWMATLWTPVRAGTDCRYGCRLLNNMTITVEREDCHGSITVTTTCAGLCE 60

Manchurian trout: 61 TTDLNYQSTWMPRSQVACNFKEWSYEKVYLEGCPGADPFFIPVAKSCDCIKCKTDNTDC 120
TTDLNYQSTW+PRSQ CNFKEWSYEKVYLEGCPG +P FIPVAKSCDCIKCKTDNTDC

Rainbow trout: 61 TTDLNYQSTWLP RSQGV CNFKEWSYEKVYLEGCPG VNP L FIPVAKSCDCIKCKTDNTDC 120

Manchurian trout: 121 DRISMATPSCV VNP LEM 137
DRISMATPSC+VNP LEM

Rainbow trout: 121 DRISMATPSCIVNP LEM 137

3) *Hippoglossus hippoglossus*(=Atlantic halibut) gonadotropin I beta

Score = 82.4 bits (202), Expect = 4e-15

Identities = 47/123 (38%), Positives = 59/123 (47%), Gaps = 4/123 (3%)

Manchurian trout: 9 LQLVIMATLWMTTPVRAGTDCWYGCRLNNMTITVEREDCHGSITITTCAGLCETTDLNYQS 68
+QLV+MAT+ AG C + CR N+ I VE I T CAG C D Y S

Atlantic halibut: 1 MQLWMATVLA AVAGAGQGCSFDCRPTNVRIPVESOGSTEYIDTTVCAGQCYNKDPVYIS 60

Manchurian trout: 69 TWMMPRSQVACNFKEWSYEKVYLEGCPGADPFFIPVAKSCDCIKCKTDNTDCDRISMATP 128
P Q +CN +WSYE ++ GCP PVA+ C C C D+TDC P

Atlantic halibut: 61 KEGPDKQNSCN-GDWSYEVKHINGCPVAVT---YPVARIHCHCSICNLDDTDCSPFP GDIP 116

Manchurian trout: 129 SCV 131
C+

Atlantic halibut: 117 GCL 119

4) *Mylopharyngodon piceus*(=Black carp) gonadotropin I beta

Score = 117 bits (294), Expect = 8e-26

Identities = 57/128 (44%), Positives = 79/128 (61%), Gaps = 3/128 (2%)

Manchurian trout: 9 LQLVIMATLWMTPVRAGTDCWYGCRLLNMTITVEREDCHGSITI—TTCAGLCETTDLNY 66
 ++V+M L +RAG++ CRL N++IT+E E+C ITI T CAGLC+T + Y
 Carp: 3 MRFVMMMLLPALMPRAGSEFRSSCRLLNISITMESEECGSCITIDTTACAGLCKTQERVY 62

Manchurian trout: 67 QSTVMPPRSQVACNFKEWSYKYLEGCPSPGADPFFI—PVAKSCDCIKCKTDNTDCDRISM 125
 +S M Q CNF+EW+YE +GCP G D F+ PVA SC+C KC +D DC +S
 Carp: 63 RSPMMQNYQNTCNFRWYTYEYFKGCPGTDVFMVVALSCECSKCNSDIADCGMLSQ 122

Manchurian trout: 126 ATPSCVVN 133
 T SC ++
 Carp: 123 QTSSCTIH 130

5) *Dicentrarchus labrax*(=sea bass) gonadotropin I beta

Score = 89.7 bits (221), Expect = 2e-17

Identities = 49/123 (39%), Positives = 66/123 (52%), Gaps = 5/123 (4%)

Manchurian trout: 9 LQLVIMATLWMTPVRAGTDCWYGCRLLNMTITVEREDCHGSITITTCAGLCETTDLNYQS 68
 +QLV+M + + RAG C +GC N++I VE I T C G C DL Y S
 sea bass: 1 MQLWMMVAV—LALARAGQGCSFGCHPTNISIQVESCGLTEVIYTTICEGQCYHEDLVYLS 59

Manchurian trout: 69 TWMPPRSQVACNFKEWSYKYLEGCPSPGADPFFIPVAKSCDCIKCKTDNTDCDRISMATP 128
 + Q CN +WSYE +++GCP G PVA++C+C C T+NTDC R P
 sea bass: 60 HYERPEQRION—GDWSYEVKHIKGPVGV—YPVARNCECTTONTENTDCGRFFPGDIP 115

Manchurian trout: 129 SCV 131
 SC+
 sea bass: 116 SCL 118

(3) LH β amino acid

1) *Oncorhynchus masou*(=Masu salmon) gonadotropin II beta subunit

Score = 303 bits (776), Expect = 3e-82

Identities = 138/142 (97%), Positives = 140/142 (98%)

Frame = +1

Manchurian trout: 1 MLGLHVGTLISLFLCILLEPVEGSLMQPCQPINQTVSLEKEGCPTCLVIQTPICSGHCVT 180
MLGLHVGTLISL LCILLEPVEGSLMQPCQPINQTVSLEKEGCPTCLVIQTPICSGHC+T
Masu salmon : 1 MLGLHVGTLISLSLCILLEPVEGSLMQPCQPINQTVSLEKEGCPTCLVIQTPICSGHCIT 60

Manchurian trout: 181 KEPVFKSPFSTVYQHVCTYRDVRYETIRLPDCPPWDPHVTPVALSCDCSLCNMDTSDC 360
KEPVF+SPFSTVYQHVCTYRDVRYE IRLPDCPPWDPHVTPVALSCDCSLCNMDTSDC
Masu salmon : 61 KEPVFRSPFSTVYQHVCTYRDVRYEMIRLPDCPPWDPHVTPVALSCDCSLCNMDTSDC 120

Manchurian trout: 361 TIESLQPDFCITQRVLTGDGMW 426
TIESLQPDFCITQRVLTGDGMW
Masu salmon : 121 TIESLQPDFCITQRVLTGDGMW 142

2) *Oncorhynchus mykiss*(=Rainbow trout) gonadotropin II beta

Score = 306 bits (785), Expect = 1e-82

Identities = 140/142 (98%), Positives = 141/142 (98%)

Manchurian trout: 1 MLGLHVGTLISLFLCILLEPVEGSLMQPCQPINQTVSLEKEGCPTCLVIQTPICSGHCVT 60
MLGLHVGTLISL LCILLEPVEGSLMQPCQPINQTVS+EKEGCPTCLVIQTPICSGHCVT
Rainbow trout: 1 MLGLHVGTLISL LLLCILLEPVEGSLMQPCQPINQTVSVEKEGCPTCLVIQTPICSGHCVT 60

Manchurian trout: 61 KEPVFKSPFSTVYQHVCTYRDVRYETIRLPDCPPWDPHVTPVALSCDCSLCNMDTSDC 120
KEPVFKSPFSTVYQHVCTYRDVRYETIRLPDCPPWDPHVTPVALSCDCSLCNMDTSDC
Rainbow trout: 61 KEPVFKSPFSTVYQHVCTYRDVRYETIRLPDCPPWDPHVTPVALSCDCSLCNMDTSDC 120

Manchurian trout: 121 TIESLQPDFCITQRVLTGDGMW 142
TIESLQPDFCITQRVLTGDGMW
Rainbow trout: 121 TIESLQPDFCITQRVLTGDGMW 142

3) *Hippoglossus hippoglossus*(=Atlantic halibut) gonadotropin II beta

Score = 161 bits (408), Expect = 5e-39

Identities = 71/114 (62%), Positives = 84/114 (73%)

Manchurian trout: 18 LEPVEGSLMQPCQPINQTVSLEKEGCPCLVIQTPICSGHCVTKEPVFKSPFSTVYQHVC 77
 L P + CQ I Q VSLEKEGCP C ++T ICSGHC TK+PV K PF VYQHVC
 Atlantic halibut: 26 LAPAVAFQLPKCQLIKQM/SLEKEGCPKCHTVETTICSGHCNTPKDPVIKIPFLNMYQHVC 85

Manchurian trout: 78 TYRDVRYETIRLPDCPPWDPHVYPVALSCDCSLCNMDTSDCTIESLQPDFCI 131
 TY+++ Y+T LPDCPP VDP V+YPVA+SC C C ++TSDCT ESLQPDFC+
 Atlantic halibut: 86 TYQELYKTFELPDCPPGVDPTVSYPVAVSCYCGRCALNTSDCTFESLQPDFCM 139

4) *Mylopharyngodon piceus*(=Black carp) gonadotropin II beta

Score = 221 bits (562), Expect = 7e-57

Identities = 97/133 (72%), Positives = 114/133 (84%), Gaps = 2/133 (1%)

Manchurian trout: 4 LHVGTLISLFLC--ILLEPVEGSLMQPCQPINQTVSLEKEGCPCLVIQTPICSGHCVTK 61
 L V ++ L C +LL + S + PC+P+N+TV++EKEGCP CLV QT ICSGHC+TK
 Carp: 2 LWRNILLLLFCYWLLVFAQSSFLPPCEPVNETVAVEKEGCPKCLVFQTTICSGHCLTK 61

Manchurian trout: 62 EPVFKSPFSTVYQH-VCTYRDVRYETIRLPDCPPWDPHVYPVALSCDCSLCNMDTSDCT 121
 EPV+KSPFSTVYQH-VCTYRDVRYET+RLPDCPP VDPH+TYPVALSCDCSLC MDTSDCT
 Carp: 62 EPVYKSPFSTVYQH-VCTYRDVRYETVRLPDCPPGVDPHITYPVALSCDCSLCTMDTSDCT 121

Manchurian trout: 122 IESLQPDFCITQR 134
 IESLQPD+C++QR
 Carp: 122 IESLQPDYCMSQR 134

5) *Dicentrarchus labrax*(=sea bass) gonadotropin II beta

Score = 176 bits (447), Expect = 1e-43

Identities = 82/131 (62%), Positives = 95/131 (71%), Gaps = 3/131 (2%)

Manchurian trout: 1 MLGLHVGTLISLFLCILLEPVEGSLMQPCQPINQTVSLEKEGCPCLVIQTPICSGHCVT 60
 +L L +G S++ L E + PCQ INQTVSLEKEGCP C ++T ICSGHC+T
 sea bass: 13 VLSLFLGATSSIWP---LATAEAFQLPPCQLINQTVSLEKEGCPKCHPVETTICSGHCIT 69

Manchurian trout: 61 KEPVFKSPFSTVYQHVMCTYRDVRYETIRLPDCPPWDPHVYTPVALSCDCSLCNMDTSDC 120
 K+PV K PFS VYQHVMCTYR+ Y+T LPDCPP VDP VTYPVA SC C C MDTSDC
 sea bass: 70 KDPVIKIPFSNVYQHVMCTYRNSHYKTFELPDCPPGVDPTVTYPVAQSCHGRCAMDTSDC 129

Manchurian trout: 121 TIESLQPDFCI 131
 T ESLQP+FC+
 sea bass: 130 TFESLQPNFCM 140

▼ Nucleotide sequence	100	96	95	74	56	56	60	59	56	58	Brachymystax lenok (열목어)
	Manchurian trout	100	99	74	57	57	60	59	56	59	Chinook Salmon
Brachymystax lenok (열목어)	100	Chinook Salmon	100	73	57	57	60	59	56	59	rainbow trout (무지개송어)
Chinook Salmon	98	100	rainbow trout	100	56	53	65	64	65	59	Oncorhynchus masou (산천어)
rainbow trout (무지개송어)	97	98	100	masu salmon	100	77	56	56	56	53	Dicentrarchus labrax (농어)
Oncorhynchus masou (산천어)	83	84	83	100	Dicentrarchus labrax	100	50	50	50	51	Atlantic halibut (넙치)
Dicentrarchus labrax (농어)	67	68	68	68	100	Atlantic halibut	100	96	95	61	grass Carp(초어)
Atlantic halibut(넙치)	66	67	67	65	84	100	grass Carp	100	95	60	Carp(잉어)
grass Carp(초어)	61	60	59	60	60	52	100	Carp	100	59	Gold fish(금붕어)
Carp(잉어)	60	58	58	60	60	61	94	100	Gold fish	100	Bubalus bubalis (소 과)
Gold fish(금붕어)	60	57	57	62	62	60	93	96	100	Bubalus bubalis	▲ Protein sequence
Bubalus bubalis (소 과)	55	57	56	61	64	60	54	53	54	100	

Fig. 5. Gonadotropin alpha (with signal sequence) and Protein Homology comparison(%)
 ※ sequence of goldfish is partial CDs .

▼ Nucleotide sequence	100	91	92	43	42	35	40	40	28	35	38	37	Brachymystax lenok (열목어)
	Brachymystax lenok	100	96	43	42	36	42	39	31	32	37	35	rainbow trout (무지개송어)
Brachymystax lenok (열목어)	100	rainbow trout	100	43	42	36	40	39	30	37	37	36	Oncorhynchus masou (산천어)
rainbow trout (무지개송어)	95	100	Oncorhynchus masou	100	85	37	66	51	31	34	39	39	Carp(잉어)
Oncorhynchus masou (산천어)	95	97	100	Carp	100	40	65	52	33	36	40	41	goldfish(붕어)
Carp(잉어)	61	61	60	100	gold fish	100	39	41	27	30	30	30	Paralichthys olivaceus(넙치)
goldfish(붕어)	61	61	60	90	100	Atlantic halibut	100	53	36	37	37	37	channel catfish (찬넬메기)
Paralichthys olivaceus(넙치)	59	59	59	51	52	100	channel catfish	100	36	42	45	40	Japanese eel
channel catfish (찬넬메기)	55	57	54	70	70	50	100	Japanese eel	100	56	52	52	Frog
Japanese eel	56	56	55	49	49	61	58	100	frog	100	65	66	Chicken
Frog	4	8	7	42	45	31	41	43	100	chicken	100	86	Mouse
Chicken	44	45	45	43	40	11	40	42	64	100	mouse	100	Human
Mouse	43	43	43	40	43	17	42	48	53	65	100	human	▲ Protein sequence
Human	43	42	42	43	44	19	43	46	46	73	84	100	

Fig. 6. FSH β (with signal sequence) and Protein Homology comparison(%)
 ※ sequence of Frog is partial CDs. Therefore, low showed homology.

▼ Nucleotide sequence	100	98	97	68	70	50	40	37	37	34	34	Brachymystax lenok (열목어)
	Brachymystax lenok	100	96	69	67	49	40	38	38	37	38	rainbow trout (무지개송어)
Brachymystax lenok (열목어)	100	rainbow trout	100	67	66	48	39	38	37	34	34	Oncorhynchus masou (산천어)
rainbow trout (무지개송어)	98	100	Oncorhynchus masou	100	87	51	43	39	42	39	40	Carp(잉어)
Oncorhynchus masou (산천어)	98	98	100	Carp	100	52	41	42	40	35	36	goldfish(금붕어)
Carp(잉어)	65	66	65	100	gold fish	100	41	37	38	34	34	Paralichthys olivaceus(넙치)
goldfish(금붕어)	60	66	67	86	100	Atlantic halibut	100	35	34	36	36	frog
Paralichthys olivaceus(넙치)	58	58	58	50	52	100	frog	100	39	36	36	chicken
frog	43	52	51	45	47	43	100	chicken	100	70	71	Human
chicken	44	44	44	45	46	42	39	100	human	100	97	Mouse
Human	42	42	42	42	42	41	36	42	100	mouse	100	Rat
Mouse	38	38	38	40	40	43	37	38	77	100	rat	▲ Protein sequence
Rat	38	39	38	41	41	45	39	39	79	95	100	

Fig. 7. LHβ (with signal sequence) and Protein Homology comparison(%)

7. 재조합형 단백질 생산

각각의 α , β 의 cloning된 fragment를 재료로 하여 호르몬 기능을 가지는 α - β 를 재조합하기 위하여 다음과 같은 strategy를 사용하였다. 재조합을 위한 primer는 양쪽 cDNA 말단으로 design하였으며, 재조합 방식은 아래 그림과 같다 (Fig. 8).

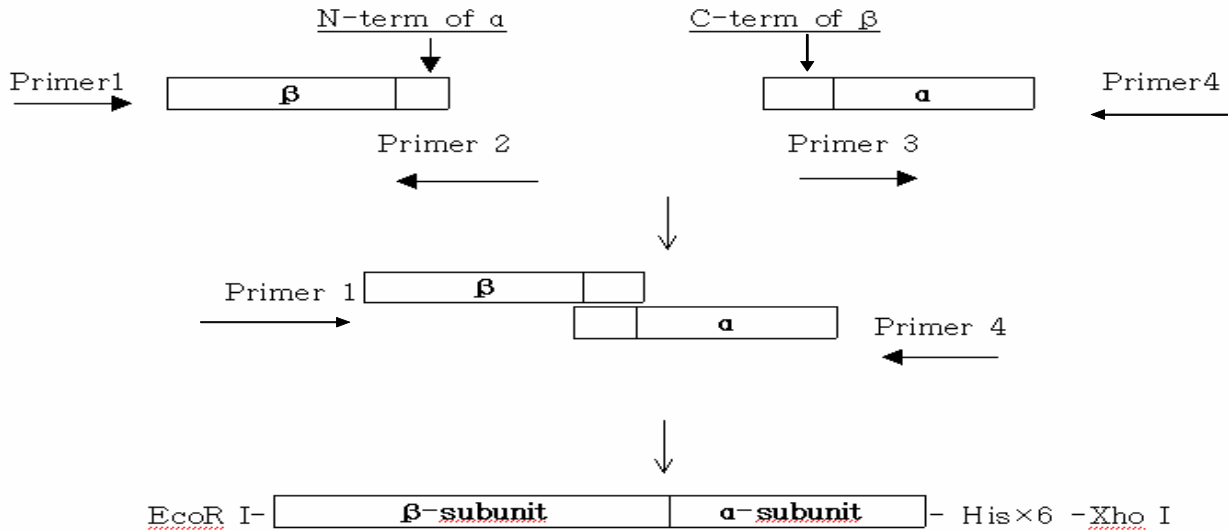


Fig. 8. Strategy for single-strand gonadotropin construction using overlapping PCR method

8. 결과

Primer 4개를 새로 디자인하여 Polymerase Chain Reaction(PCR)을 실행하여 α / β 를 재조합하였다. α 의 부분은 단백질이 합성을 시작하는 mature sequence를 시작으로 primer를 제작하였다. α 의 C-term에는 유전자 재조합된 α / β 융합된 단백질을 용이하게 추출하기 위하여 His \times 6를 붙여 primer를 제작하였다(Ho *et al.*, 1989; Min, 2001). 각각을 PCR하여 재조합된 α / β 는 발현벡터에 ligation하여 plasmid DNA를 추출하여 insert의 유무를 확인한 후 sequencing을 하였다. sequencing 결과는 아래와 같다 (Figs. 10, 11).

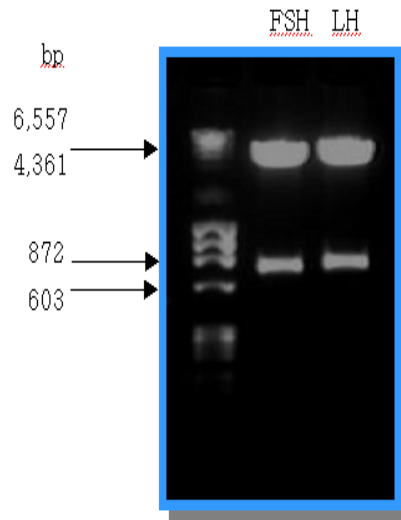


Fig. 9. Confirmation for gonadotropin β - α insertion

가. Nucleotide sequence : FSH β -GTH α

GAATTCATGTA CTGCACCCACTTAAAGACGCTGCAGCTGGTCATCATGGCAACGCTGTG
 GATGACACCAGTGAGGGCAGGGACAGACTGCTGGTATGGCTGCCGACTAAACAACATGA
 CCATCACCGTGGAGAGAGAGGACTGTCACGGAAGCATCACCATCACCACCTGCGCCGGCC
 TGTGCGAAACGACGGATCTGAACTACCAGAGCACATGGATGCCACGCTCCCAGGTGGCG
 TGTA ACTTCAAGGAGTGGTCCTACGAGAAGGTCTACCTGGAAGGCTGTCCATCCGGGGC
 CGACCCCTTCTTCATTCTGTTGCCAAGAGCTGCGATTGCATCAAATGCAAGACTGACA
 ACACCGACTGTGATCGCATAAGCATGGCAACACCCAGCTGCGTAGTAAACCCACTAGAA
 ATG/TATCCAAACAGTGACAAGACAAACATGGGCTGTGAGGAATGCACACTGAAGCCGAA
 CACAATCTTCCCCAACATCATGCAGTGTACAGGCTGCTGCTTCTCCAGAGCTTATCCAA
 CCCACTACGGTCCAAGCAAACCATGCTGGTCCCCAAGAACATCACCTCTGAAGCCACGT
 GCTGCGTTGCAAAAGAAGGGGAAAGGGTCACCACCAAGGATGGCTTCCCGGTGACGAAC
 CACACAGAGTGTCACTGCAGCACCTGTTATTACCATAAATCAcatcatcatcatcatTAA**CTC**
GAG

↳. Amino acid sequence: FSH β -GTH α

```

7 atgtactgcacccacttaagacgctgcagctggtcatcatggca
  M Y C T H L K T L Q L V I M A
52 acgctgtggatgacaccagtgagggcagggacagactgctggtat
  T L W M T P V R A G T D C W Y
97 ggctgccgactaaacaacatgaccatcacctggagagagaggac
  G C R L N N M T I T V E R E D
142 tgtcacggaagcatcacatcaccacctgcgcccggcctgtgcgaa
  C H G S I T I T T C A G L C E
187 acgacggatctgaactaccagagcacatggatgccacgctcccag
  T T D L N Y Q S T W M P R S Q
232 gtggcgtgtaactcaaggagtggcctacgagaaggctacactg
  V A C N F K E W S Y E K V Y L
277 gaaggctgtccatccggggccgaccccttctcattcctgttgcc
  E G C P S G A D P F F I P V A
322 aagagctgcgattgcatcaaagcaagactgacaacaccgactgt
  K S C D C I K C K T D N T D C
367 gatcgcataagcatggcaacacccagctgcgtagtaaaccacta
  D R I S M A T P S C V V N P L
412 gaaatgatccaaacagtgacaagacaacatgggctgtgaggaa
  E M Y P N S D K T N M G C E E
457 tgcacactgaagccgaacacaatctccccaacatcatgacagtg
  C T L K P N T I F P N I M Q C
502 acaggctgctgcttctccagagcttatccaacccactacggctc
  T G C C F S R A Y P T P L R S
547 aagcaaaccatgctggcctcccaagaacatcacctctgaagccacg
  K Q T M L V P K N I T S E A T
592 tgctgcgttgcaaaagaaggggaaagggcaccaccaaggatggc
  C C V A K E G E R V T T K D G
637 ttcccggtgacgaaccacacagagtgcactgcagcacctgttat
  F P V T N H T E C H C S T C Y
682 taccataaatcacatcatcatcatcatcattaa 714
  Y H K S H H H H H H H *

```

Fig. 10. Open reading frame of recombinant Manchurian trout FSH(N-His-FSH β /GTH α).

Black boxes - EcoR I(F), Xho I (R); Underline - start of a subunit;

Lower letters - His \times 6

ㄷ. Nucleotide sequence : LHβ-GTHα

GAATTCATGTTAGGTCTTCATGTAGGCACCTTGATCTCCCTGTTCTGTGCATCCTCCTG
 GAACCCGTTGAGGGGTCTTCATGCAGCCCTGTCAGCCCATCAACCAGACTGTGTCTCTGG
 AGAAGGAAGGCTGCCAACGTGCTTAGTCATTCAAACCCCTATCTGCAGTGGCCACTGCGT
 CACCAAGGAGCCGGTTTTCAAGAGCCATTTTCCACCGTGTACCAGCATGTGTGCACCTAC
 CGAGACGTCCGCTATGAAACGATCCGCCTACCTGACTGTCCCCCTTGGGTGGACCCTCATG
 TCACCTACCCTGTGGCTCTGAGCTGTGACTGCAGCCTCTGTAACATGGACACTTCTGACTG
 TACCATCGAGAGCCTGCAGCCAGACTTCTGCATTACCCAAAGAGTACTAACGGATGGTGAC
 ATGTGG/TATCCAAACAGTGACAAGACAAACATGGGCTGTGAGGAATGCACACTGAAGCCG
 AACACAATCTTCCCCAACATCATGCAGTGTACAGGCTGCTGCTTCTCCAGAGCTTATCCAA
 CCCCCTACGGTCCAAGCAAACCATGCTGGTCCCCAAGAACATCACCTCTGAAGCCACGTG
 CTGCGTTGCAAAAGAAGGGGAAAGGGTCACCACCAAGGATGGCTTCCCGGTGACGAACCAC
 ACAGAGTGTCACTGCAGCACCTGTTATTACCATAAATCAcatcatcatcatcatTAA**CTCGAG**

ㄹ. Amino acid sequence : LHβ-GTHα

1 atgtaggtcttcatgtaggcaccttgatctccctgttcctgtgc
 M L G L H V G T L I S L F L C
 46 atcctcctggaacccggtgaggggtctctc**atg**cagccctgtcag
 I L L E P V E G S L M Q P C Q
 91 cccatcaaccagactgtgtctctggagaaggaaggctgcccaacg
 P I N Q T V S L E K E G C P T
 136 tgcttagtcattcaaacccctatctgcagtgccactgctcacc
 C L V I Q T P I C S G H C V T
 181 aaggagccggtttcaagagcccattttccaccgtgtaccagcat
 K E P V F K S P F S T V Y Q H
 226 gtgtgcacctaccgagacgtccgctatgaaacgatccgcctacct
 V C T Y R D V R Y E T I R L P
 271 gactgtccccctgggtggaccctcatgtcacctaccctgtggct
 D C P P W V D P H V T Y P V A
 316 ctgagctgtgactgcagcctctgtaac**atg**gacacttctgactgt
 L S C D C S L C N M D T S D C
 361 accatcgagagcctgcagccagacttctgcattacccaagagta
 T I E S L Q P D F C I T Q R V
 406 ctaacggatggtgac**atg**tggtatccaacagtgacaagacaac
 L T D G D M W Y P N S D K T N
 451 **atg**ggctgtgaggaatgcacactgaagccgaacacaatctcccc
 M G C E E C T L K P N T I F P
 496 aacatc**atg**caggttacaggctgctgcttctccagagcttatcca
 N I M Q C T G C C F S R A Y P
 541 accccactacgggtccaagcaaacc**atg**ctggtcccccaagaacatc
 T P L R S K Q T M L V P K N I
 586 acctctgaagccacgtgctgctgctgcaaaagaaggggaaagggct
 T S E A T C C V A K E G E R V
 631 accaccaaggatggcttcccgggtgacgaaccacacagaggtcac
 T T K D G F P V T N H T E C H
 676 tgcagcacctgttattaccataaatcacatcatcatcatcatcat
 C S T C Y Y H K S H H H H H H
 721 **taa** 723
 *

Fig. 11. Open reading frame of recombinant Manchurian trout LH(N-His-LHβ/GTHα).

Black boxes - EcoR I(F), Xho I (R); Underline - start of a subunit; Lower letters - His×6

9. 아미노기 말단에 His-Tag을 가지는 cDNA를 cloning

기존의 열목어 GTH α , LH β cDNA를 이용하여 분비형 즉 성숙형 LH호르몬의 아미노기 말단영역에 His \times 6 -Tag를 갖는 유전자재조합을 two-step PCR 방법으로 제작하였다(Ho *et al.*, 1989; Min, 2001) (Fig.12).

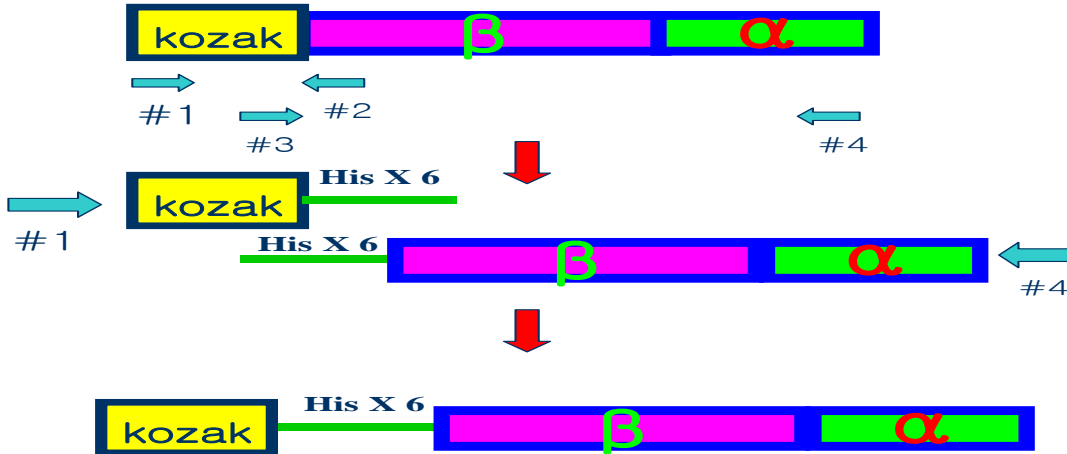


Fig. 12. PCR amplification of N-His \times 6-LH cDNA

Table 2. Oligo primers used in this study.

Primer	Direction	sequence
LH β -EcoRI Kozak (Primer 1)	sence	5'-GCGAATTCACCATGGTAGGTCTTCATGTAGG-3'
mtLH β -NHis-R (Primer 2)	antisence	5'-GAGATGATGATGATGATGATGAGACCCCTCAACGGGTTCCAG-3'
mtLH β -NHis-F (Primer 3)	sence	5'-TCTCATCATCATCATCATCATCTCATGCAGCCCTGTCAGCCC-3'
GP α -R (Primer 4)	antisence	5'-CTCGAGTTATGATTTATGGTAATAACA-3'

가. 아미노기 말단에 His-Tag을 가지는 열목어 LH cDNA cloning

단백질의 추출 효율을 증가시키기 위하여 성숙호르몬의 아미노기 말단에 His-Tag를 가지는 LH construct를 제작하여, pcDNA3 vector에 삽입하였으며 염기서열을 결정하였다.

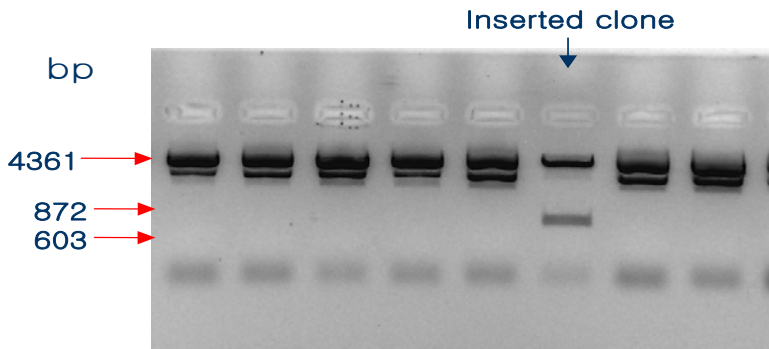


Fig. 13. Confirmation for gonadotropin insertion (EcoR I/Xho I)

나. Sequencing 결과

```

atggttaggtcttcatgtaggcaccttgatctccctgttctctgtgc
M V G L H V G T L I S L F L C
atcctcctggaaaccggttgagggtctcatcatcatcatcatcat
I L L E P V E G S H H H H H H
catctcatgcagccctgtcagcccacatcaaccagactgtgtctctg
H L M Q P C Q P I N Q T V S L
gagaaggaaggctgcccacgtgcttagtcattcaaaccctatc
E K E G P T C L V I Q T P I
tgcagtggccactgcgtcaaccaaggagccggttttcaagagccca
C S G H C V T K E P V F K S P
ttttccaccgtgtaccagcatgtgtgcacctaccgagacgtccgc
F S T V Y Q H V C T Y R D V R
tatgaaacgatccgcctacctgactgtcccccttgggtggaccct
Y E T I R L P D C P E W V D P
catgtcacctaccctgtggctctgagctgtgactgcagcctctgt
H V T Y P V A L S C D C S L C
aacatggacacttctgactgtaccatcgagagcctgcagccagac
N M D T S D C T I E S L Q P D
tctgcattacccaaagagtactaacggatggtagcatgtggtat
F C I T Q R V L T D G D M W Y
ccaaacagtgacaagacaacatgggctgtgaggaatgcacactg
P N S D K T N M G C E E C T L
aagccgaacacaatcttccccaacatcatgcagtgtacaggctgc
K P N T I F P N I M Q C T G C
tgcttctccagacttatccaaccctactacggtccaagcaaacc
C F S R A Y P T P L R S K Q T
atgctggtccccaagaacatcacctctgaagccacgtgctgcgtt
M L V P K N I T S E A T C C V
gcaaaaagaagggaagggtcaccaccaaggatggcttcccgggtg
A K E G E R V T T K D G F P V
acgaaccacacagagtgtcactgcagcacctgttattaccataaa
T N H T E C H C S T C Y Y H K
tcataa
S *

```

Fig. 14. Open reading frame of recombinant Manchurian trout LH(N-His-LHβ/GTHα).

다. 신호 펩타이드와 성숙호르몬의 분해영역의 해석

신호 펩타이드와 성숙호르몬의 분해는 23-24번 아미노산 G-S 사이임이 Signal IP 예측시스템을 통하여 분석 가능하였음(Fig. 15.).

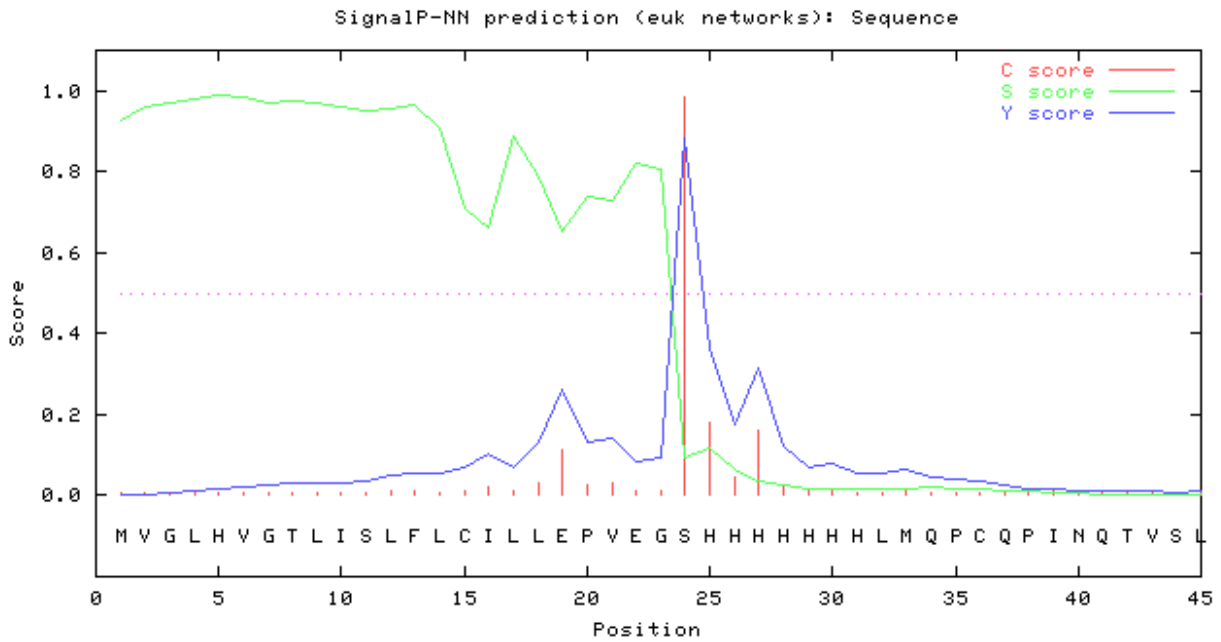


Fig. 15. Signal peptide and mature hormone prediction program

```
# data
>Sequence          length = 45
# Measure  Position  Value  Cutoff  signal peptide?
max. C     24         0.983  0.32   YES
max. Y     24         0.881  0.33   YES
max. S     5          0.989  0.87   YES
mean S     1-23       0.881  0.48   YES
D          1-23       0.881  0.43   YES
# Most likely cleavage site between pos. 23 and 24: VEG-SH
```

10. 열목어 갑상선 자극 호르몬 유전자의 cloning

생식선 자극 호르몬과 공통적인 GTH α 쇠와 호르몬 특이적인 TSH β 쇠로 구성된 열목어 갑상선 자극 호르몬(TSH) β 쇠를 cloning하여 생식선 자극 호르몬과의 비교 실험에 사용하고자 하였다.

가. 재료 및 방법

실험 샘플 : 열목어의 뇌하수체 cDNA pool

primer 제작 : 무지개송어 TSH β subunit에 기초

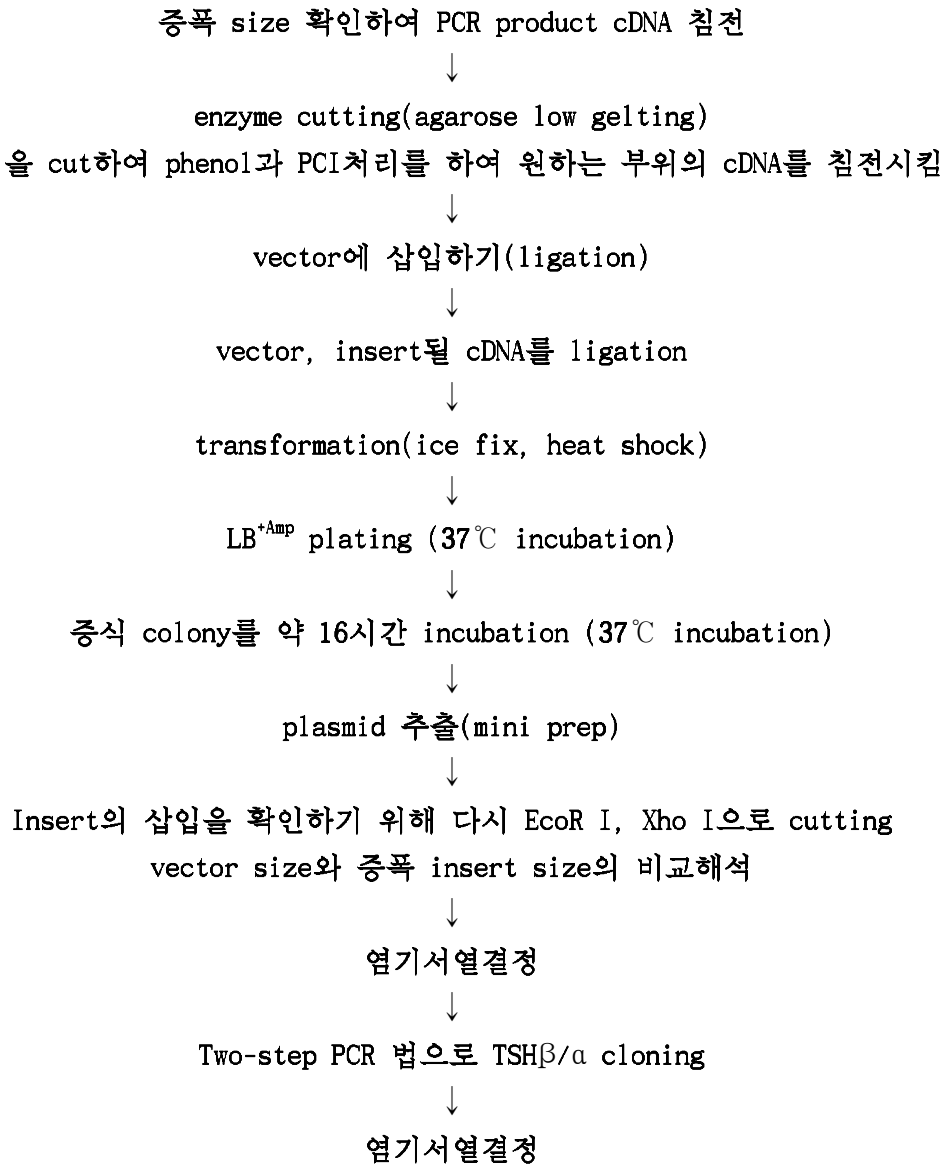
Forward : 5'-ATGGAATTGTCGGTGGCC-3'

Reverse : 5'-CTACATCCACCCCAACTGA-3'

PCR

denaturation	94 °C	30s	
annealing	50 °C	30s	30cycles
extension	72 °C	30s	

전기영동 : 1.5% 의 agarose gel 사용, cDNA size 확인



나. nucleotide 와 amino acid의 서열 해석결과

(1) 열목어 TSHβ subunit Open Reading Frame 분석

```

1 atggaattgtccgtggccatgtgtggtctcctttgctgctcttc
  M E L S V A M C G L L C L L F
46 agccaagctgtgcccattgtgtgcccacagactacactctgtat
  S Q A V P M C V P T D Y T L Y
91 gaggagagacgtgaatgtgacttctgctgtccatcaatacgacc
  E E R R E C D F C V S I N T T
136 attgcatgggcttctgctactcaagggacagtaacatgaaggag
  I C M G F C Y S R D S N M K E
181 ctggccggaccgcttctccttatccagagaggctgtacctatgat
  L A G P R F L I Q R G C T Y D
226 cagtgaggataccgaacagtcatactacctggctgcccgtccat
  Q V E Y R T V I L P G C P L H
271 gccaaccctctcttcacctaccccgctggccctcagctgccactgt
  A N P L F T Y P V A L S C H C
316 ggcacctgcaacacagacagtgatgagtgtgccacaaaggccagc
  G T C N T D S D E C A H K A S
361 agtggagacggcgccaggtgtccaagccactcagacacatctac
  S G D G A R C S K P L R H I Y
406 ccataccctggccggaacaactacatccaccccaactga      444
  P Y P G R N N Y I H P N *
    
```

다. 열목어와 NCBI에 등록된 nucleotide 및 amino acid sequence 비교

(1) nucleotide sequence 비교

1)>[gi|435639|dbj|D14692.1|ONHTBS](#)**U**

Oncorhynchus mykiss(=Rainbow trout)mRNA for thyrotropin beta-subunit

Score = 801 bits (404), Expect = 0.0

Identities = 434/444 (97%)

Strand = Plus / Plus

```

Manchurian trout: 1 atggaattgtccgtggccatgtgtggtctcctttgctgctcttcagccaagctgtgccc 60
  |||
    
```

```

Rainbow trout : 58 atggaattgtccgtggccatgtatggtctcctttgctgctcttcagccaagctgtgccc 117
    
```

Manchurian trout: 61 atgtgtgtgcccacagactacactctgtatgaggagagacgtgaatgtgacttctgcgtg 120
|||||

Rainbow trout : 118 atgtgtgtgcccacggattacactctgtatgaggagagacgtgaatgtgacttctgcgtg 177

Manchurian trout: 121 tccatcaatagcaccatttgcattggcttctgctactcaagggacagtaacatgaaggag 180
|||||

Rainbow trout: 178 gccatcaatagcaccatttgcattggcttctgctactcaagggacagtaacatgaaagag 237

Manchurian trout: 181 ctggccggaccgcggttccttatccagagaggctgtacctatgatcaggtggagtaccga 240
|||||

Rainbow trout : 238 ctggccggaccacggttccttatccagagaggctgtacctatgaccaggtggagtaccga 297

Manchurian trout: 241 acagtcatactacctggctgcccgtccatgccaaccctctcttcacctaccccgtggcc 300
|||||

Rainbow trout : 298 acagtcatactacctggtgcccgtccatgccaaccctctcttcacctaccccgtggcc 357

Manchurian trout: 301 ctgagctgccactgtggcacctgcaacacagacagtgatgagtggtgcccacaaggccagc 360
|||||

Rainbow trout : 358 ctgagctgccactgtggcacctgcaacacagacagtgatgagtggtgcccacaaggccagc 417

Manchurian trout: 361 agtggagacggcgccagggtgtccaagccactcagacacatctaccataccctggccgg 420
|||||

Rainbow trout : 418 agtggagacggcgccagggtgtccaagccactcagacacatctaccataccctggcctg 477

Manchurian trout: 421 aacaactacatccaccccaactga 444
||||

Rainbow trout : 478 aacagctacatccaccccaactga 501

2)>[gi|3885466|gb|AF060566.1|U](#)

Salmo salar(=Atlantic salmon) thyrotropin beta subunit mRNA

Score = 785 bits (396), Expect = 0.0

Identities = 433/444 (97%), Gaps = 1/444 (0%)

Strand = Plus / Plus

Manchurian trout: 1 atggaattgtccgtggccatgtgtggtctcctttgctgctcttcagccaagctgtgccc 60
 |||
Salmo salar : 35 atggaattgtccgtggccatgtgtggtctcctttgctgctcttcagccaagctgtgccc 94

Manchurian trout: 61 atgtgtgtgcccacagactacactctgtatgaggagagacgtgaatgtgacttctgcgtg 120
 |||
Salmo salar : 95 atgtgtgtgcccacggactacactctgtatgaggagagacgtgaatgtgacttctgcgtg 154

Manchurian trout: 121 tccatcaatacagaccatttgcattgggcttctgctactcaagggacagtaacatgaaggag 180
 |||
Salmo salar : 155 gccatcaatacagaccatttgcattgggcttctgctactcaagggacagtaacatgaaggag 214

Manchurian trout: 181 ctggccggaccgcttcccttatccagagaggctgtacctatgatcaggtggagtaccga 240
 |||
Salmo salar : 215 ctggccggaccacgcttcccttatccagagaggctgtacctatgaccaggtggagtaccga 274

Manchurian trout: 241 acagtcatactacctggctgcccgctccatgccaacctctcttcacctaccccgtagcc 300
 |||
Salmo salar : 275 acagtcatactacctggctgcccactccatgccaacctctcttcacctaccccgtagcc 334

Manchurian trout: 301 ctcagctgccactgtggcacctgcaacacagacagtgatgagtggtgcccacaaggccagc 360
 |||
Salmo salar : 335 ctcagctgccactgtggcacctgcaacacagacagtgatgagtggtgcccacaaggccagc 394

Manchurian trout: 361 agtggagacggcgccagggttccaagccactcagacacatctaccataccctggccgg 420
 |||
Salmo salar : 395 agtggagacggcgccagggttccaagccactcagacacatcta-ccataccctggcctg 453

Manchurian trout: 421 aacaactacatcccccaactga 444
 |||
Salmo salar : 454 aacaactacatcccccaactga 477

3) [gi|431912|emb|X73493.1|AATBS](#)

A. anguilla(=European eel) mRNA for thyrotropin beta-subunit

Score = 79.8 bits (40), Expect = 4e-12

Identities = 70/80 (87%)

Strand = Plus / Plus

Manchurian trout: 76 gactacactctgtatgaggagagacgtgaatgtgacttctgcgtgtccatcaatcagacc 135
 ||| | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |

A. anguilla : 145 gactacacgctgtacgtggagaaacctgagtgacttctgcgtggccatcaacaccacc 204

Manchurian trout: 136 attgcatgggcttctgcta 155

|| | | | | | | | | | | | | | | | | | |

A. anguilla : 205 atctcatgggcttctgcta 224

4) [gi|30313577|gb|AY158008.1|](#)

Anguilla japonica(=Japanese eel) thyroid stimulating hormone beta subunit

Score = 71.9 bits (36), Expect = 1e-09

Identities = 69/80 (86%)

Strand = Plus / Plus

Manchurian trout: 76 gactacactctgtatgaggagagacgtgaatgtgacttctgcgtgtccatcaatcagacc 135
 ||| | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |

Anguilla japonica : 641 gactacacgctgtacgtggagaaaccagagtgacttctgcgtggccatcaacaccacc 700

Manchurian trout: 136 attgcatgggcttctgcta 155

|| | | | | | | | | | | | | | | | | | |

Anguilla japonica: 701 atctcatgggcttctgcta 720

5) [gi|46093511|dbj|AB175833.1|](#)

Anguilla japonica(=Japanese eel) mRNA for thyroid-stimulating hormone beta

Score = 71.9 bits (36), Expect = 1e-09

Identities = 69/80 (86%)

Strand = Plus / Plus

Manchurian trout: 76 gactacactctgtatgaggagagacgtgaatgtgacttctgcgtgtccatcaatacgacc 135
 ||||| | |||| | |||| | | ||||| || || ||||| ||||| || |||

Anguilla japonica: 121 gactacacgctgtacgtggagaaaccagagtgacttctgcgtggccatcaacaccacc 180

Manchurian trout: 136 atttgcattgggcttctgcta 155
 || ||||| ||||| |||||

Anguilla japonica: 181 atctgcattgggcttctgcta 200

라. Amino acid sequence 비교

1) >[gi|586132|sp|P37240|TSHB_ONCMY](#)

Thyrotropin beta chain precursor (Thyroid-stimulating hormone beta subunit) (TSH-beta) (TSH-B)

[gi|477151|pir||A48194](#)

thyrotropin beta chain - rainbow trout

[gi|435640|dbj|BAA03518.1|](#)

Oncorhynchus mykiss(=Rainbow trout) thyrotropin beta-subunit

Score = 314 bits (804), Expect = 4e-85

Identities = 143/147 (97%), Positives = 145/147 (98%)

Frame = +1

Manchurian trout: 1 MELSVAMCGLLCLLFSQAVPMCPTDYTLYEERRECDFCVSIINTTICMGFCYSRDSNMKE 180
 MELSVAM GLLCLLFSQAVPMCPTDYTLYEERRECDFCV+INTTICMGFCYSRDSNMKE

Rainbow trout : 1 MELSVAMYGLLCLLFSQAVPMCPTDYTLYEERRECDFCVAINTTICMGFCYSRDSNMKE 60

Manchurian trout: 181 LAGPRFLIQRGCTYDQVEYRTVILPGCPLHANPLFTYPVALSCHOGTCNTDSDECAHKAS 360
 LAGPRFLIQRGCTYDQVEYRTVILPGCPLHANPLFTYPVALSCHOGTCNTDSDECAHKAS

Rainbow trout : 61 LAGPRFLIQRGCTYDQVEYRTVILPGCPLHANPLFTYPVALSCHOGTCNTDSDECAHKAS 120

Manchurian trout: 361 SGDGARCSKPLRHIYPYPGRNNYIHPN 441
 SGDGARCSKPLRHIYPYPG N+YIHPN

Rainbow trout : 121 SGDGARCSKPLRHIYPYPGLNSYIHPN 147

2)>[gi|6136056|sp|073824|TSHB_SALSA](#)

Thyrotropin beta chain precursor (Thyroid-stimulating hormone beta subunit)(TSH-beta) (TSH-B)
[gi|3091282|gb|AAC77908.1|](#)

Salmo salar(=Atlantic Salmon) thyrotropin beta subunit

Score = 293 bits (751), Expect = 6e-79

Identities = 134/135 (99%), Positives = 135/135 (100%)

Frame = +1

Manchurian trout: 1 MELSVAMCGLLCCLLFSGQAVPMCVPTDYTYEERRECDFCVSIINTTICMGFCYSRDSNMKE 180
MELSVAMCGLLCCLLFSGQAVPMCVPTDYTYEERRECDFCV+INTTICMGFCYSRDSNMKE
Salmo salar : 1 MELSVAMCGLLCCLLFSGQAVPMCVPTDYTYEERRECDFCVAINTTICMGFCYSRDSNMKE 60

Manchurian trout: 181 LAGPRFLIQRGCTYDQVEYRTVILPGCPLHANPLFTYPVALSCHOGTCNTDSDECAHKAS 360
LAGPRFLIQRGCTYDQVEYRTVILPGCPLHANPLFTYPVALSCHOGTCNTDSDECAHKAS
Salmo salar : 61 LAGPRFLIQRGCTYDQVEYRTVILPGCPLHANPLFTYPVALSCHOGTCNTDSDECAHKAS 120

Manchurian trout: 361 SGDGARCSKPLRHIY 405
SGDGARCSKPLRHIY
Salmo salar : 121 SGDGARCSKPLRHIY 135

3)>[gi|11359153|gb|AAD51753.2|](#)

Aristichthys nobilis(=striped bighead) thyrotropin beta precursor

Score = 218 bits (556), Expect = 2e-56

Identities = 95/138 (68%), Positives = 114/138 (82%)

Frame = +1

Manchurian trout: 25 GLLCCLLFSGQAVPMCVPTDYTYEERRECDFCVSIINTTICMGFCYSRDSNMKELAGPRFLI 204
G+L LL A PMC PT+YT+Y ER+EC++CV++NTTICMGFC+SRDSN+KEL GPRFL+
Aristichthys nobilis: 8 GMLGLLMKIAAPMCAPTEYTYIERQEONCVAVNTTICMGFCFSRDSNMKELVGPFRFLV 67

Manchurian trout: 205 QRGCTYDQVEYRTVILPGCPLHANPLFTYPVALSCHOGTCNTDSDECAHKASSGDGARCS 384
QRGCTY +VEYRT ILPGCP +A+P FTYPVALSCHC TCNT SDECAHK S+ +CS
Aristichthys nobilis: 68 QRGCTYQVEYRTAILPGOPSYADPHFTYPVALSCHCSTCNTHSDECAHKTSNA-ARKCS 126

Manchurian trout: 385 KPLRHIYPYPGRNNYIHP 438

KP+RH+YP N+YI P

Aristichthys nobilis:127 KPVRHLYPDHEENSYIQP 144

⌘}. Manturian trout TSH β / α construct in pcDNA3

```
1 atggaattgtccgtggccatgtgtggtctcctttgctgctcttc
  M E L S V A M C G L L C L L F
46 agccaagctgtgcccagtggtgtgcccacagactacactctgtat
  S Q A V P M C V P T D Y T L Y
91 gaggagagacgtgaatgtgacttctgctgtcaatcaatacgacc
  E E R R E C D F C V S I N T T
136 atttgcatgggcttctgctactcaagggacagtaacatgaaggag
  I C M G F C Y S R D S N M K E
181 ctggccggaccgcgtttccttatccagagaggctgtacctatgat
  L A G P R F L I Q R G C T Y D
226 caggtggagtaccgaacagtcatactacctggctgcccgtccat
  Q V E Y R T V I L P G C P L H
271 gccaacctctcttcacctaccccgaggccctcagctgccactgt
  A N P L F T Y P V A L S C H C
316 ggcacctgcaacacagacagtgatgagtggtgcccacaaggccagc
  G T C N T D S D E C A H K A S
361 agtggagacggcgccaggtgttccaagccactcagacacatctac
  S G D G A R C S K P L R H I Y
406 ccataccctggcgggaacaactacatccacccaactatccaac
  P Y P G R N N Y I H P N Y P N
451 agtgacaagacaaaacatgggctgtgaggaatgcacactgaagccg
  S D K T N M G C E E C T L K P
496 aacacaatcttcccaacatcagtcagtgtagggctgctgcttc
  N T I F P N I M Q C T G C C F
541 tcagagcttatccaacccactacgggtccaagcaaaccatgctg
  S R A Y P T P L R S K Q T M L
586 gtccccaagaacatcacctctgaagccacgtgctgctgctgcaaaa
  V P K N I T S E A T C C V A K
631 gaaggggaaagggtcaccaccaaggatggcttcccgggtgacgaac
  E G E R V T T K D G F P V T N
676 cacacagagtgctactgcagcacctgttattaccataatcaccat
  H T E C H C S T C Y Y H N H H
721 catcatcatcatcattaa 735
  H H H H H *
```

11. 열목어 유전자재조합 FSH 및 LH cDNA constructs

제1차 년도 및 2차 년도에서 재조합한 DNA constructs의 세포 내 단백질 발현을 및 생산된 단백질의 안정성을 증가시키기 위하여 다음과 같은 방법으로 개량하였다(Klein *et al.*, 2003) (Figs. 16, 17).

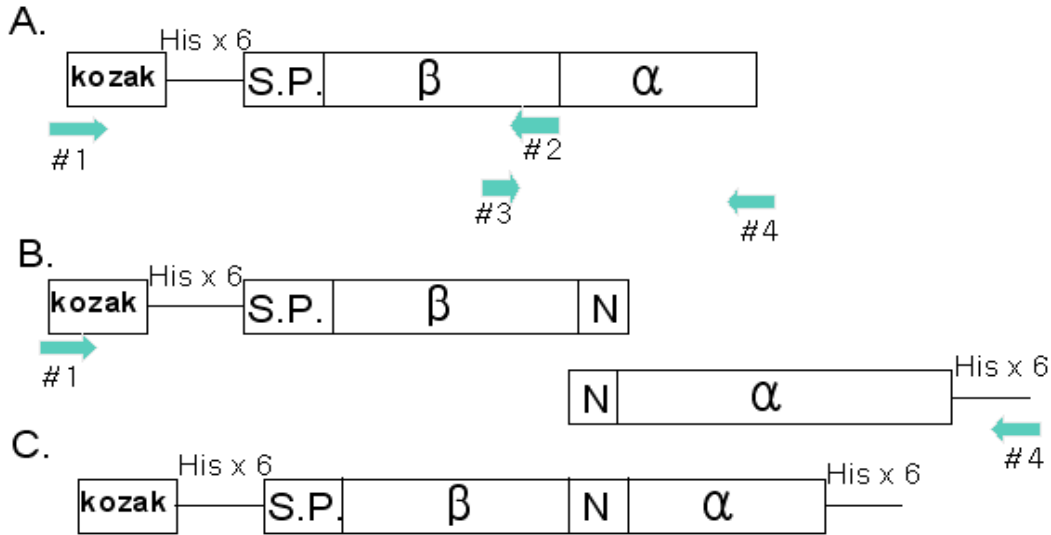


Fig.16. Recombinant DNA constructs of Manchurian trout FSH and LH by overlapping PCR method. A : template cDNA ; B : overlapping PCR ; C : recombinant DNA S.P. : signal peptide; N : NCS (N-linked carbohydrate sequence)

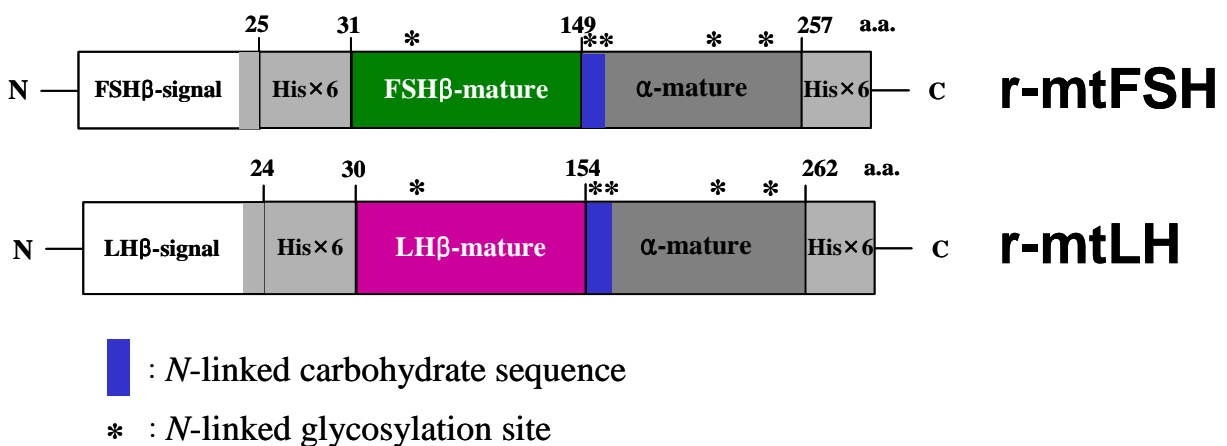


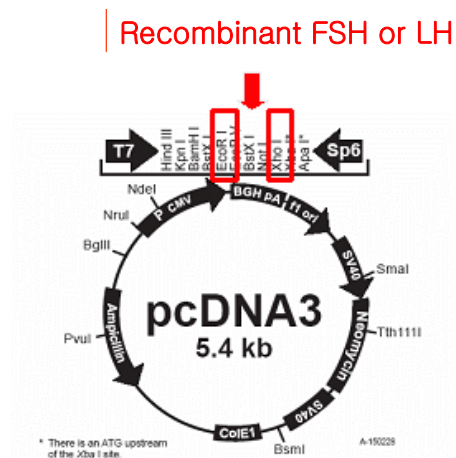
Fig. 17. Schematic diagram of DNA constructions for r-mtFSH and r-mtLH proteins.

Table. 3. Oligo primers used in this study.

Primer	Direction	Sequence
msFSH β -F EcoR I Kozak (#1)	Forward	5'-GCG AAT TCA CCA TGG ACT GCA CCC ACT TAA AG-3'
msLH β -F EcoR I Kozak(#1)	Forward	5'-GCG AAT TCA CCA TGG TAG GTC TTC ATG TAG G-3'
mtFSH β -NCS-R (#2)	Reverse	5'- <u>CTG AAC CTG TCG CAT TTG ATC CAC TCA TTT CTA GTG</u> GGT TTA CTA CG-3'
mtLH β -NCS-R (#2)	Reverse	5'- <u>CTG AAC CTG TCG CAT TTG ATC CAC TCC ACA TGT CAC</u> CAT CCG TTA-3'
mtGTH α -NCS-F (#3)	Forward	5'- <u>AGT GGA TCA AAT GCG ACA GGT TCA GGT TCT AAC GCC</u> <u>ACT TCA GGT TCT TAT CCA AAC AGT GAC AAG AC-3'</u>
GP α His-R (New)(#4)	Reverse	5'-CCG CTC GAG TTA ATG ATG ATG ATG ATG ATG TGA TTT ATG GTA-3'

Note. Underlines indicate NCS (N-linked carbohydrate sequence).
Shadowbox indicates poly-histidine residues.

(A)



(B)

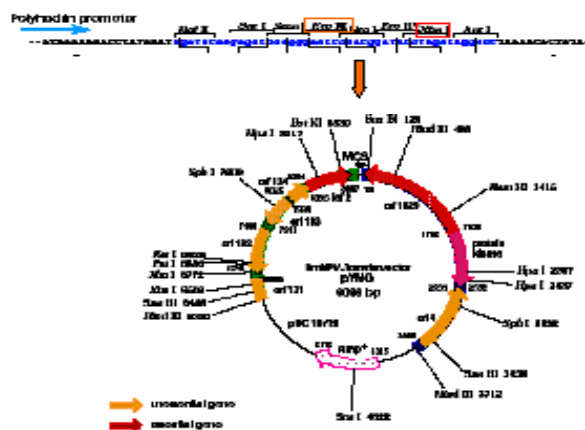


Fig. 18. Cloning vectors for CHO-K1 cells (A) and insect cells (B).

가. FSH 및 LH cDNA constructs의 확인 및 염기서열

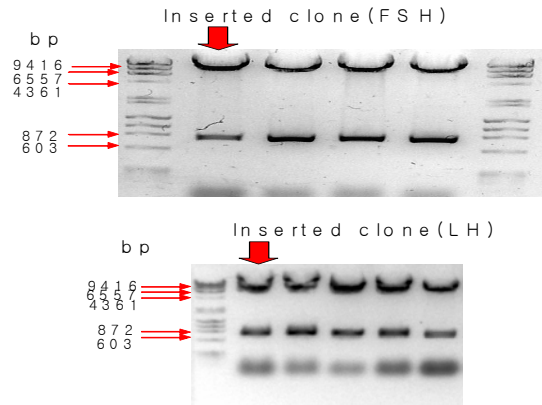


Fig. 19. Confirmation of FSH and LH recombination in pYNG vector. The inserted DNAs were confirmed by restriction enzyme treatment (EcoR I and Xba I).

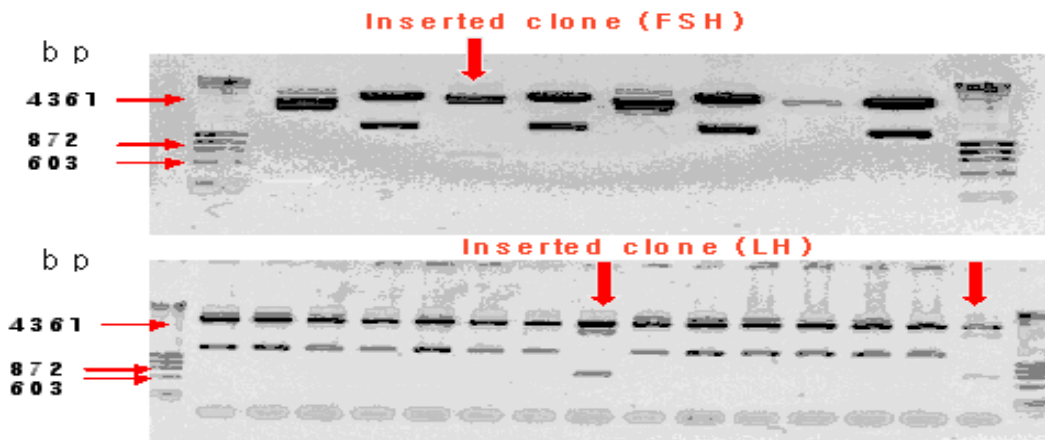


Fig. 20. Confirmation of FSH and LH recombination in pcDNA3 vector. The inserted DNAs were confirmed by restriction enzyme treatment (EcoR I and Xho I).

```

10 atggactgcaccacttaaagacgctgcagctggctcttcatggca
   M D C T H L K T L Q L V F M A
55 acgctgtggatgacaccagtgagggcagggcatcatcatcatcat
   T L W M T P V R A G H H H H H
100 catacagactgctggatggctgccgactaaacaacatgaccatc
   H T D C W Y G C R L N N M T I
145 accgtggagagagaggactgtcacggaagcatcaccatcaccacc
   T V E R E D C H G S I T I T T
190 tgcgccggcttgtgcgaaacgacggatctgaactaccagagcaca
   C A G L C E T T D L N Y Q S T
235 tggatgccacgctcccagggtggcgtgtaacttcaaggagtgggtcc
   W M P R S Q V A C N F K E W S
280 tacgagaaggtctacctggaaggctgtccatccggggccgacccc
   Y E K V Y L E G C P S G A D P
325 ttcttcattcctgttgccaagagctgcgattgcatcaaatgcaag
   F F I P V A K S C D C I K C K
370 actgacaacaccgactgtgatcgcataagcatggcaacaccagc
   T D N T D C D R I S M A T P S
415 tgcgtagtaaaccactagaagtgagtggatcaaatgcgacaggt
   C V V N P L E V S G S N A T G
460 tcaggttctaacgccacttcaggttcttatccaaacagtgacaag
   S G S N A T S G S Y P N S D K
505 acaaacatgggctgtgaggaatgcacactgaagccgaacacaatc
   T N M G C E E C T L K P N T I
550 ttccccaacatcatgcagtgtagcaggctgctgcttctccagagct
   F P N I M Q C T G C C F S R A
595 tatccaaccctacaggtccaagcaaaccatgctgggtccccaaag
   Y P T P L R S K Q T M L V P K
640 aacgtcacctctgaagccacgtgctgctgcaaaaagaaggggaa
   N V T S E A T C C V A K E G E
685 agggtcaccaccaaggatggnttcccggtgacgaaccacacagag
   R V T T K D G F P V T N H T E
730 tgtcactgcaagcacctgttattaccataaatcacatcatcatcat
   C H C S T C Y Y H K S H H H H
775 catcattaa 789
   H H *

```

Fig. 21. Open reading frame of recombinant Manchurian trout FSH.
Red characters : signal peptide region; yellow characters : Histidine tag; green characters :
N-linked carbohydrate sequence (NCS).


```

17 atggtaggcaccttgatctccctggtcctgtgcatcctcctggaa
    M V G T L I S L F L C I L L E
62 cccgttgaggggtctcatcatcatcatcatcatctcatgcag
    P V E G S H H H H H H H L M Q
107 ccctgtcagcccatcaaccagactgtgtctctggagaaggaaggc
    P C Q P I N Q T V S L E K E G
152 tgcccaacgtgcttagtcattcaaaccctatctgcagtgccac
    C P T C L V I Q T P I C S G H
197 tgcgtcaccaaggagccggttttcaagagcccattttccaccgtg
    C V T K E P V F K S P F S T V
242 taccagcatgtgtgcacctaccgagacgtccgctatgaaacgatc
    Y Q H V C T Y R D V R Y E T I
287 cgcctacctgactgtcccccttgggtggaccctcatgtcacctac
    R L P D C P P W V D P H V T Y
332 cctgtggctctgagctgtgactgcagcctctgtaacatggacact
    P V A L S C D C S L C N M D T
377 tctgactgtaccatcgagagcctgcagccagacttctgcattacc
    S D C T I E S L Q P D F C I T
422 caaagagtactaaccggatggtgacatgtggagtggatcaaatgcg
    Q R V L T D G D M W S G S N A
467 acaggttcaggttctaaccgacttcaggttcttatccaaacagt
    T G S G S N A T S G S Y P N S
512 gacaagacaaacatgggctgtgaggaatgcacactgaagccgaac
    D K T N M G C E E C T L K P N
557 acaatcttccccaacatcatgcagtgtagcaggctgctgcttctcc
    T I F P N I M Q C T G C C F S
602 agagcttatccaacccccactacgggtccaagcaaaccatgctggtc
    R A Y P T P L R S K Q T M L V
647 cccaagaacatcacctctgaagccacgtgctgcgttgaaaagaa
    P K N I T S E A T C C V A K E
692 ggggaaanggtcaccaccaaggatggntttcccggtgacgaacca
    G E X V T T K D G F P G D E P
737 cacagaagtgtcactgcagcacctggttattaccataaatcacatc
    H R S V T A A P V I T I N H I
782 attcatcatcatcatcattaa 805
    I H H H H H H H *

```

Fig. 22. Open reading frame of recombinant Manchurian trout LH.
Red characters : signal peptide region; yellow characters : Histidine tag; green characters :
N-linked carbohydrate sequence (NCS).

5절. 생식선 자극 호르몬의 유전공학적 생산

1. 열목어 생식선 자극 호르몬 유전자의 세포내 형질 도입 및 발현

- 형질 도입 호르몬 유전자산물의 SDS-PAGE 및 Western blotting 검사

가. 유전자발현에 사용된 세포주

본 연구에 사용된 세포주 (CHO-K1; Fig. 23)는 서울 대학교 암 연구소 한국 세포주 은행 (KOREAN CELL LINE BANK, KCLB)으로부터 입수하였으며, 구입 후 액체질소에 동결 보존하였으며, 일부는 계대 배양하여 실험에 사용하였다.

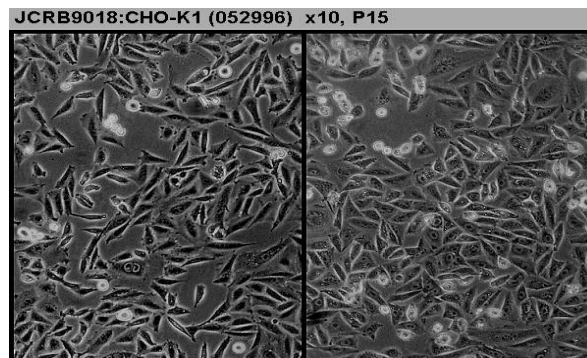


Fig. 23. CHO-K1 used in this study.

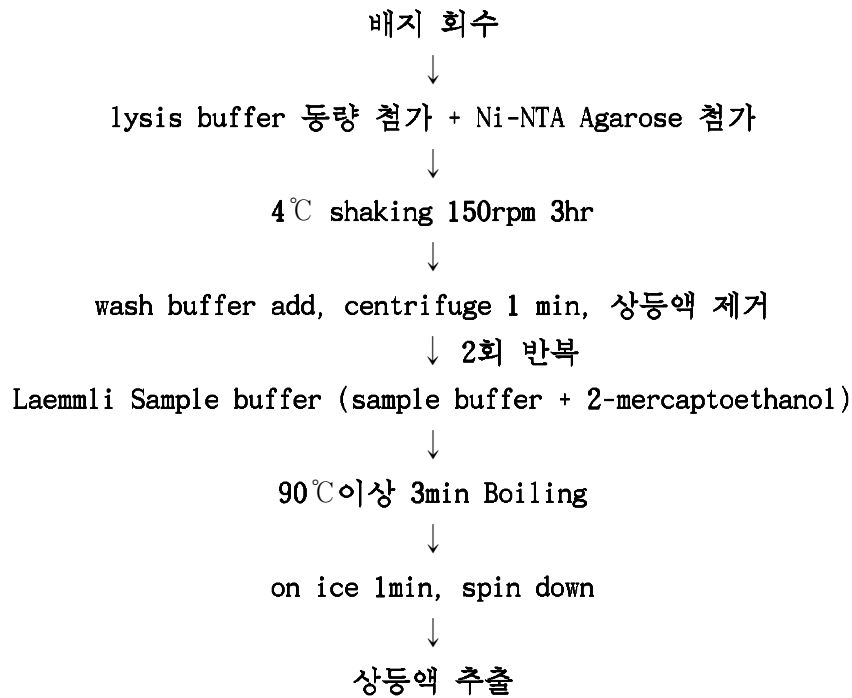
나. 호르몬 분자의 형질 도입 방법

형질도입 유도체로서 liposome complex-based chemical (Lipofectamin, Invitrogen)을 다음과 같이 사용하였다.

	24 well based transfection	10 cm based transfection
배지, 온도, CO ₂ 농도	10% FBS포함 Ham's F-12, 37°C, 5%	10% FBS포함 Ham's F-12, 37°C, 5%
세포밀도	50,000 cells	1,000,000 cells
배지교환	seeding 후 24시간	seeding 후 24시간
DNA농도	1 ug	10 ug
Lipofectamin	4 ul	20 ul
Lipofectamin+ DNA 첨가 후 배양시간	3 시간	3 시간
배지교환	Lipofectamin첨가 후 3시간째	Lipofectamin첨가 후 3시간째
배양시간	~ 4일	~ 20일

2. 호르몬 분자의 확인방법

가. 단백질의 Affinity 추출



나. SDS-PAGE

Running gel

- 30.8 : 2.7% acrylamide : bis(monomer solution)
- 4X Running Gel buffer (1.5M Tri-Cl, pH 8.8)
- 10% SDS
- APS (10% Ammonium Persulfate)
- TEMED
- DDW

Stacking gel

- 30.8 : 2.7% acrylamide : bis(monomer solution)
- 4X Stacking Gel buffer (0.5M Tri-Cl, pH 6.8)
- 10% SDS
- APS (10% Ammonium Persulfate)
- TEMED
- DDW

다. Western Blot

SDS PAGE에 단백질 loading

↓

80V 1hr 30min

↓

160V 1hr

SDS PAGE를 membrane에 옮김.
 (Transfer buffer 사용, 15V 400mA 35min)
 ↓
 TBS-T buffer 에 membrane washing
 (shaking 5min)
 ↓ 2회 반복
 membrane에 TBS-T (5% skim milk) 첨가
 ↓ 1hr shaking, RT
 His-probe (First antibody) 첨가
 ↓ 4°C shaking 16hr~
 membrane washing (TBS-T shaking 5min)
 ↓ 2회 반복
 membrane에 TBS-T (5% skim milk) 첨가
 +
 goat anti-rabbit Ig G -HRP(secondary antibody)첨가
 (1 : 1000 비율)
 ↓ shaking 1hr(RT)
 TBS-T washing (shaking 5min)
 ↓ 3회 반복
 암실 현상

3. 열목어 생식선 자극 호르몬 유전자를 가진 세포주의 개발

가. screening 방법

(Selection of cells stably expressing the CHO-K1 Kozak NHis-FSH/ CHO-K1 Kozak NHis-LH)

Seeding 2×10^5 CHO-K1 cells in a $\phi 100$ mm dish

↓ 1 day

Transfection with FSH/LH by Lipofectamin method to CHO-K1 at 70% confluence

↓ 1 day

medium change(HAM'S F-12, 10% FBS, 1000ug/ml genitacin)

↓ 3 days

seeding at cell density 400 cells/dish into selective medium

↓ 9days, incubation without motion

Cell cloning by (penicillin) Cup method

↓

Harvested, re-seeded and cultured in a 24 well of
16 of growing genitacin resistant colonies/FSH
10 of growing genitacin resistant colonies/LH

↓ 3 days

Harvested, re-seeded and cultured in a Φ 35mm dish

↓ 3 days

Extraction of DNA constructs by cell lysis

Detection of DNA constructs(FSH/LH) in the transfected cells by western blot analysis

나. 호르몬의 생산 및 추출

<5절.2-가. 단백질의 Affinity 추출> 참조

다. 결과

(1) 열목어 FSH 호르몬의 SDS-PAGE 후 Western blot 분석

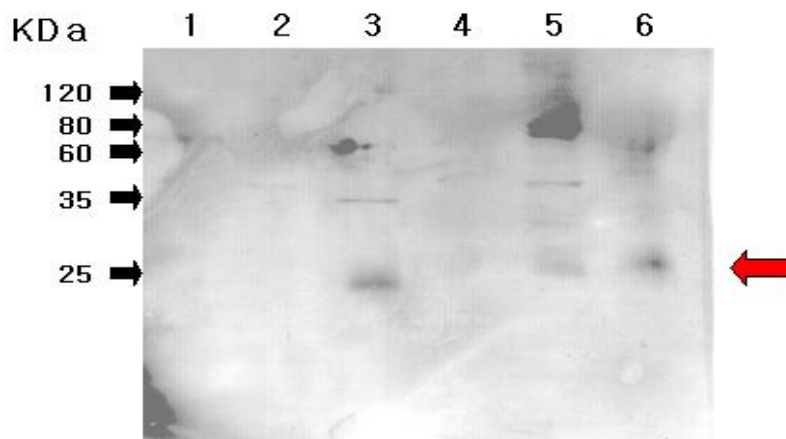


Fig. 24. Western blot on FSH production in CHO-K1 cells

Lane 1: Cell lysate at 0 hr after transfection (cell lysate) ; Lane 2: culture medium at 0 hrs; Lane 3: cell lysate at 24 hrs; Lane 4: culture medium at 24 hrs; Lane 5: cell lysate at 48 hrs; Lane 6: culture medium at 48 hrs.

(2) 열목어 LH 호르몬의 SDS-PAGE 분석

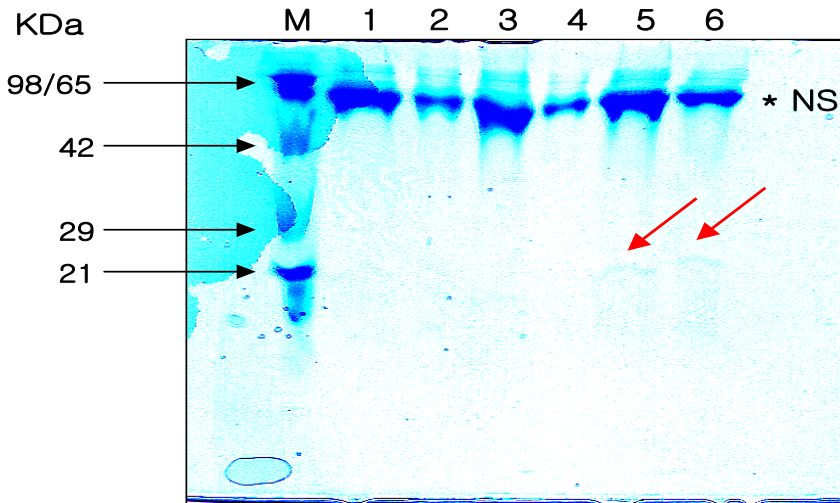


Fig. 25. A result on LH production in CHO-K1 cells by SDS-PAGE analysis

Lane 1: culture medium at 0 hr after transfection Lane 2: culture medium at 12 hrs; Lane 3: culture medium at 24 hrs; Lane 4: culture medium at 36 hrs; Lane 5: culture medium at 48 hrs; Lane 6: culture medium at 60 hrs.

4. 연어 생식선자극호르몬 수용체 유전자를 가진 세포주의 개발

가. screening 방법

(Selection of cells stably expressing the COS7-sFSHR/ COS7-LHR)

Seeding 2×10^6 COS7 cells in a $\phi 100$ mm dish

↓ 1 day

Transfection with FSHR/pcDNA3(genitacin resistant gene)(20/1) by Lipofectamin method to COS7 at 70% confluence

↓ 1 day

Passage cells at 1:50,1:20,1:5 into seletive medium(DMEM, 10% FBS, 1000ug/ml genitacin)

↓ 6 days

Medium change (DMEM, 10% FBS, 600ug/ml genitacin)

↓ 9 days, incubation without motion

Cell cloning by (penicillin) Cup method

↓

Harvested, re-seeded and cultured in a 24 well of

21 of growing genitacin resistant colonies/FSHR

12 of growing genitacin resistant colonies/LHR

↓ 3 days

Harvested, re-seeded and cultured in a $\phi 35$ mm dish

↓ 3 days

Extraction of DNA constructs by cell lysis

Detection of DNA constructs(FSHR/LHR) in the transfected cells by PCR

나. 결과

Stable cell line 제조, 확인 (COS7-sFSHR)

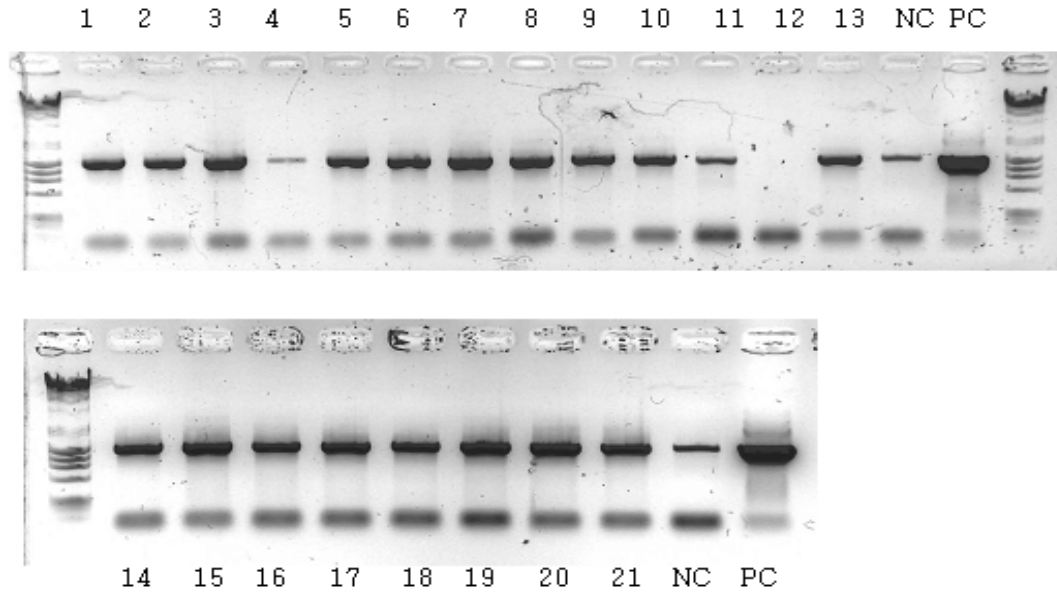


Fig. 26. PCR results to determine salmon FSH receptor expression in COS-7 cell
NC: CHO-K1-LH PC: sFSHR (2, 11, 13, 14, 15, 16, 17 cell stored)

Stable cell line 제조, 확인 (COS7-sLHR)

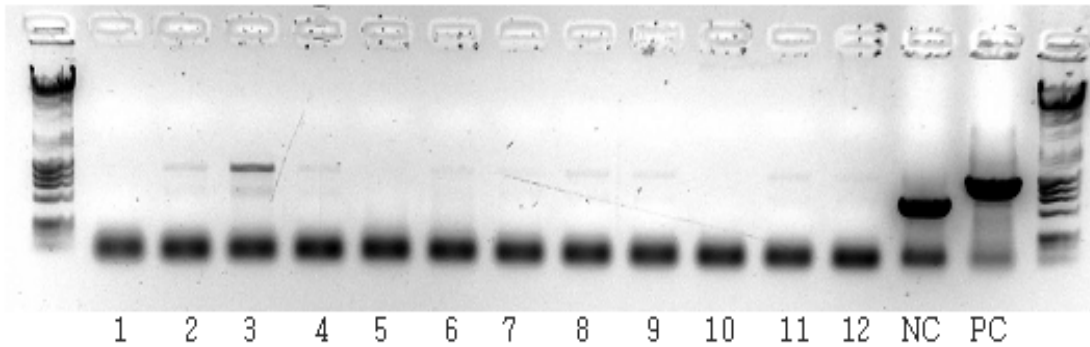


Fig. 27. PCR results to determine salmon LH receptor expression in COS-7 cell
NC: COS7-FSHR PC: sLHR (3, 6 cell stored)

5. 열목어 생식선자극호르몬 대량분비세포의 선택 및 정제

가. FSH 및 LH 발현용 Stable cell (CHO-K1) cloning

-cell culture 100 ϕ dish, 배지 8ml.

-DNA : mtFSH/LH 10 μ g

-transfection : Lipofectamin 15 μ l(Qiagen, Germany)

-3hr 후 배지 교환.(Ham's F-12 medium(10% Fetal bovine serum, 1X Antibiotics) + GENITICIN(1000 μ g/ml)

-2일 후 세포 수 희석.(Ham's F-12 medium(10% Fetal bovine serum, 1X Antibiotics) + GENITICIN(600 μ g/ml)

-10일 후 cell colony형성 확인.

-cell cloning

-24well plate에 각각의 cell colony를 배양.

-60 ϕ dish에 배양.

-cell 저장. 배지는 회수하여 단백질이 분비되었는지 확인.

나. Suspension culture (Large culture)

(1) 사 용 : Stable Cell (Kozak NHis-NCS LH)

: SPINNER FLASKS

: 자석 교반기

: CHO-S-SFM II

: 37 $^{\circ}$ C CO₂ (8%)INCUBATOR

(2) 순 서 : stable cells 해동(HAM'S F-12 100%)

: SPINNER FLASK (HAM'S F-12 100%)

: SPINNER FLASK (HAM'S F-12 50% + CHO-S-SFM II 50%)

: SPINNER FLASK (HAM'S F-12 25% + CHO-S-SFM II 75%)

: SPINNER FLASK (HAM'S F-12 12.5% + CHO-S-SFM II 87.5%)

: SPINNER FLASK (CHO-S-SFM II 100%)

(G418 : 300 μ g/ml)

(3) 1 단계 : stable cell 안정화

-부착된 상태에서 HAM's F-12 배지로 2~3차례 계대 해줌.

(4) 2 단계 : suspension culture

-spinner flask를 사용하여 부유된 상태로 세포를 배양함.

- HAM's F-12 100% + GENITICIN(300 μ g/ml)

(3 X 10⁵ cells/ml \rightarrow 5X10⁵ cells/ml)

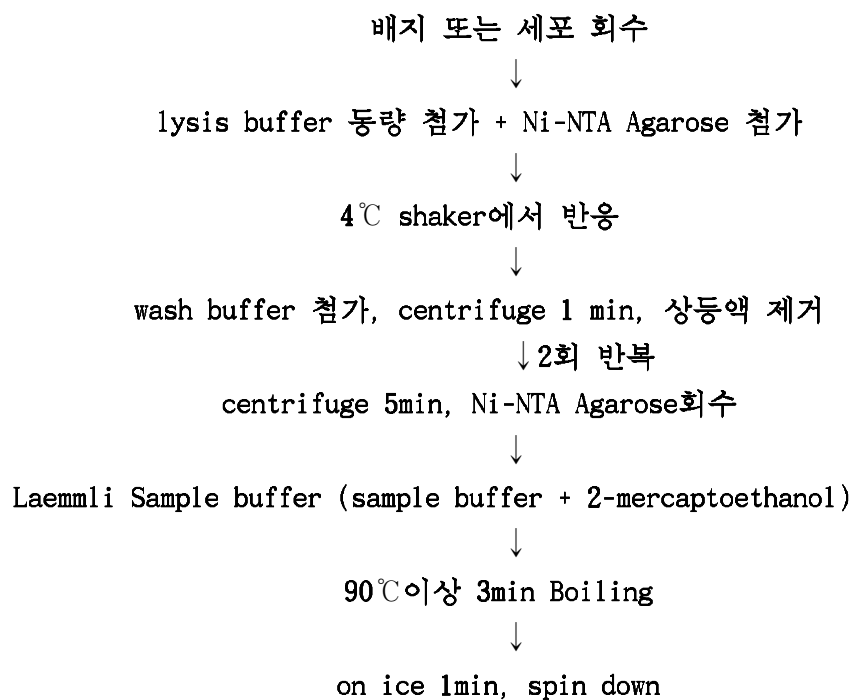
(5) 3 단계 : suspension culture

- CHO-S-SFM II 배지의 양을 늘리면서 새 배지에 적응.
- 100% CHO-S-SFM II 가 되었을 때 FBS 0.1% 부터 적응.
- FBS의 양이 0%가 될 때까지 적응.
- 3×10^5 cells/ml로 계대.
- 2일 후 3×10^6 cells/ml 로 자람. 배지와 cell 회수.
- 회수한 배지에 0.2mM PMSF 처리.
- Ni-NTA agarose 를 사용하여 단백질 정제.
- Western blot을 통해 확인.



Fig. 28. Large culture system for production of recombinant FSH and LH from CHO-K1 cells using spinner flask.

(6) 단백질 확인



↓
상등액 추출
샘플 일부를 Western blot 방법으로 단백질 확인

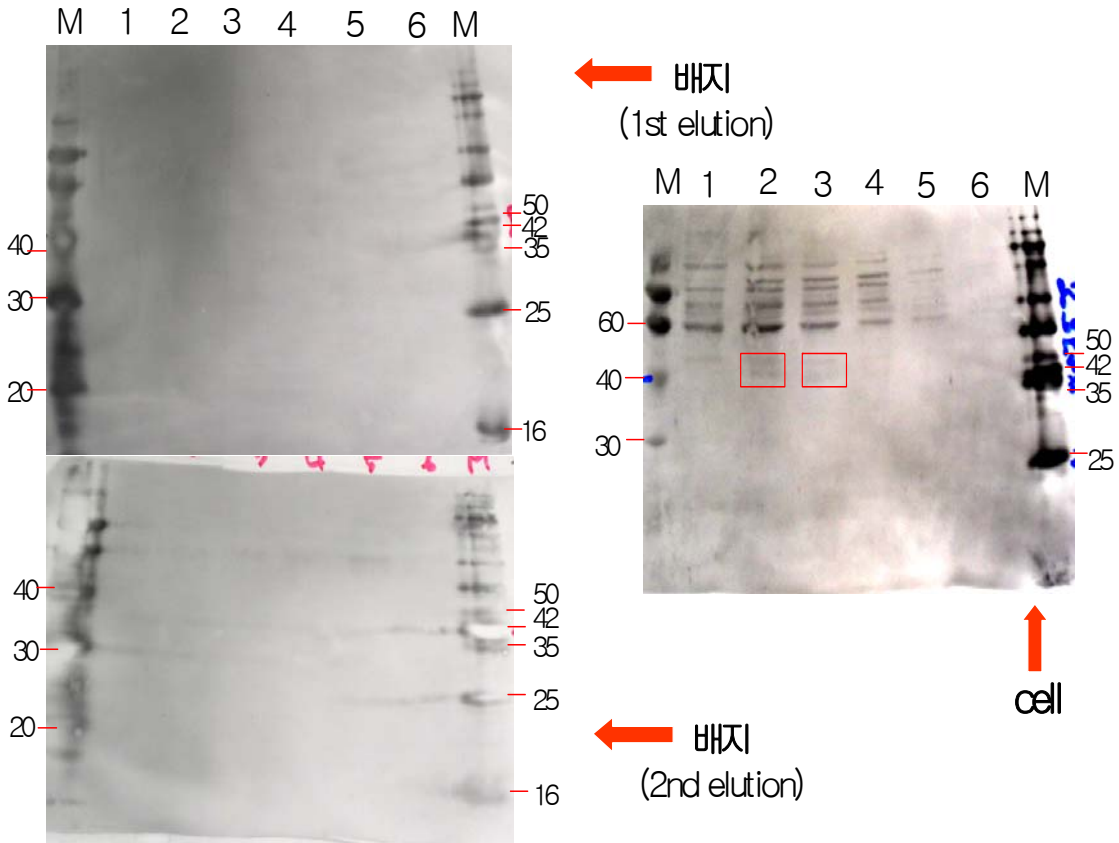


Fig. 29. Western blot analyses using His-Tag antibody on FSH and LH proteins in CHO-K1 cells
Left panel (upside) : eluted FSH fractions from CHO-S-SFM II medium (Lane 1 - 6); (downside)
: eluted LH fractions from CHO-S-SFM II medium (Lane 1 - 6); Right panel : eluted LH fractions
from cell lysates (Lane 1- 6).

- 유전자재조합단백질은 세포질 내에 농축되어 있음.
CHO-K1 세포의 배양 시간을 장기간 요함.

라. baculovirus-silkworm larvae system을 이용한 단백질 대량 생산

- cloning된 pYNG transfer vector 대량 정제.
- Baculovirus에 형질 전환.
- Baculovirus를 silkworm에 감염.
- 6일~7일 후 hemolymph를 채취.
- Ni-NTA agarose bead 사용하여 단백질 정제.
- Western blot 확인.

(1) 재조합단백질의 affinity 대량정제

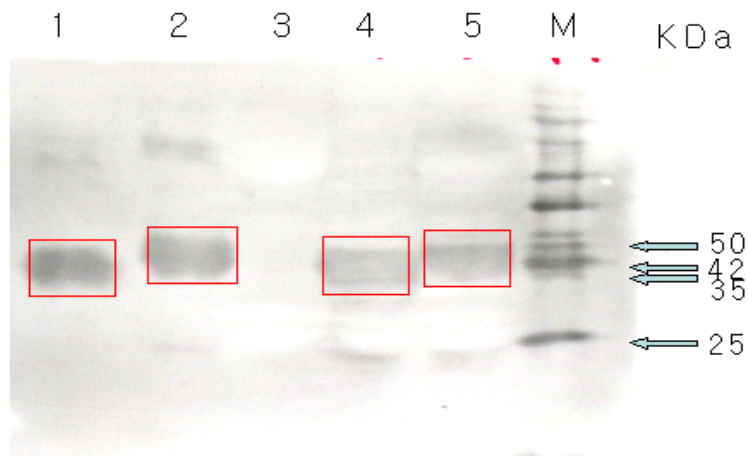
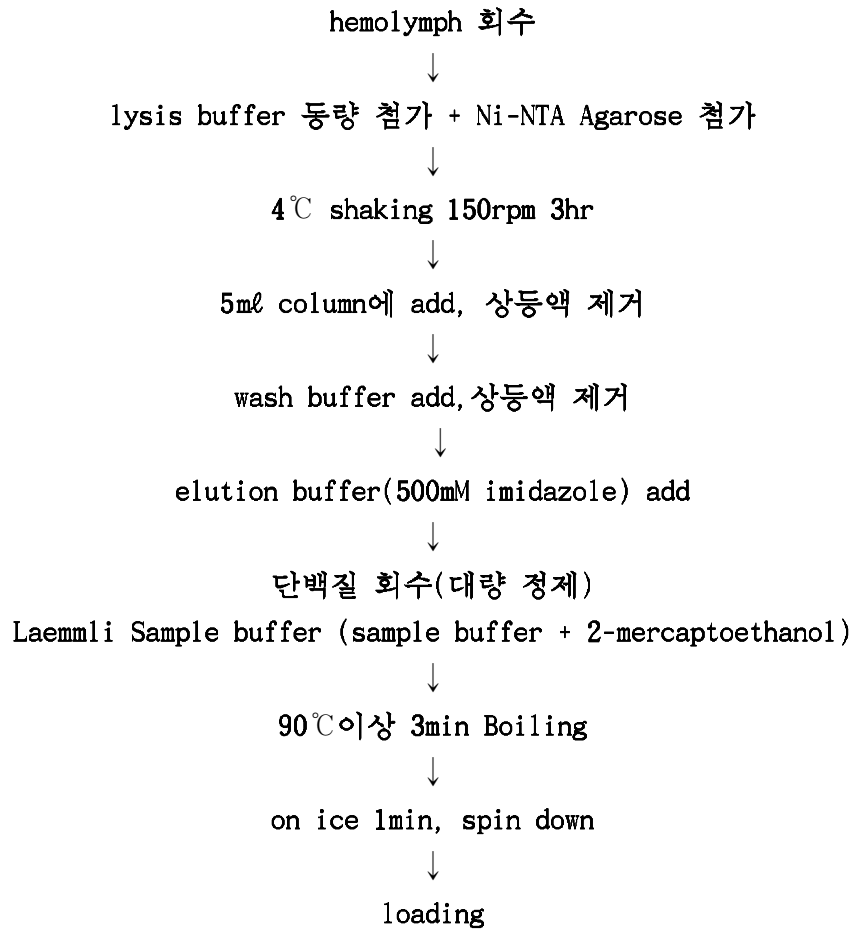


Fig. 30. Western blot analyses using HisTag antibody on FSH and LH proteins in silk worm hemolymph.

Lane 1 : purified FSH; 2 : purified LH; 3 : silk worm hemolymph; 4 : hemolymph expressing FSH; 5 : hemolymph expressing LH; M : EBM-1017 western marker

- 유전자 재조합 단백질은 hemolymph로 분비되며 각각의 단백질생산량은 0.3 - 0.6 mg으로 추정됨 (UV-spectrophotometer 측정).

마. De-N-glycosylation

(1) 실험 목적

: 당쇄 수식이 단백질의 안정성을 유지시켜주는 것으로 알려져 있기 때문에 유전자재조합단백질에 수식되어 있는 당쇄를 제거시켰을 때의 분자크기를 조사하고자 함.

(2) 실험방법

: N-glycosidase F (PNGase F)를 사용하여 정제한 단백질의 N-glycosylation 부위를 제거 후 Western blot 실험(Kamei H *et al.*, 2003).

(3) De-N-glycosylation

- 단백질과 동량의 denaturing buffer (0.45% SDS, 1M Tris-HCl(pH8.0), 0.1M 2-mercaptoethanol) 처리.
- 100°C heat, 3min.
- 100mU N-glycosidase F 처리.
- 37°C 15 hr incubation

kDa

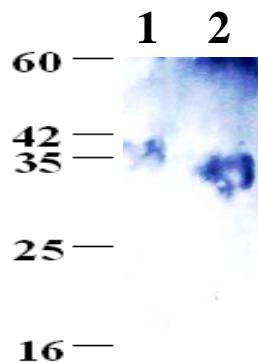


Fig. 31. Lowered molecular weight of r-mtFSH by de-N-glycosylation. Lane 1, control protein; lane 2, N-glycosidase F treated protein.

- De-N-glycosylation 처리된 FSH의 경우 35kDa의 크기에서 약 32kDa으로 크기가 작아진 것을 western blot으로 확인하였으며, 당 수식의 제거로 인한 단백질의 분자량에 차이가 생긴 것으로 판단됨.

6절. 생식선 자극 호르몬의 생물학적 활성 조사

1. *In vitro* bioassay

정제된 유전자재조합단백질의 기능을 확인하기 위하여

- 1) 단백질(호르몬)이 수용체와 결합한 후, 세포내 cAMP농도를 측정하여 세포내 신호전달이 일어났는지를 조사.
- 2) 호르몬을 직접 난모세포에 노출시켜서 난모세포의 여포세포에서 방출된 스테로이드 호르몬의 농도를 측정하여 스테로이드호르몬생합성을 유도하는지 조사.

가. 간접법 (reporter assay)

(1) 도입 세포 : COS-7 cell

(2) 도입 방법 : CaCl_2

(3) receptor : amago salmon FSH/LH-수용체 (sFSH/LH-R)

(4) reporter gene : cyclic AMP-responsive element-regulated luciferase expression plasmid (pCRE-luc)

(5) 실험 방법

24well plate에 세포 계대.

하루 배양(CO_2 incubator(5%), 37°C)

transfection	pCRE-luc (200ng/ μl)	1.0 μl
	β -gal(pcDNA3) (200ng/ μl)	1.0 μl
	sFSH/LH-R (200ng/ μl)	1.0 μl
	2.5M CaCl_2	2.5 μl
	DDW	19.5 μl
	2X BBS	25.0 μl
	total	50.0 μl

vortex.

spin down.

RT. 20min.

각 well에 50 μl 씩 첨가.

18hr 후 배지 교환과 함께 호르몬 처리. (10^{-1} , 1, 10 $\mu\text{g}/\text{ml}$)

5~8hr 사이에 cell lysis. (2hr내에 측정.)

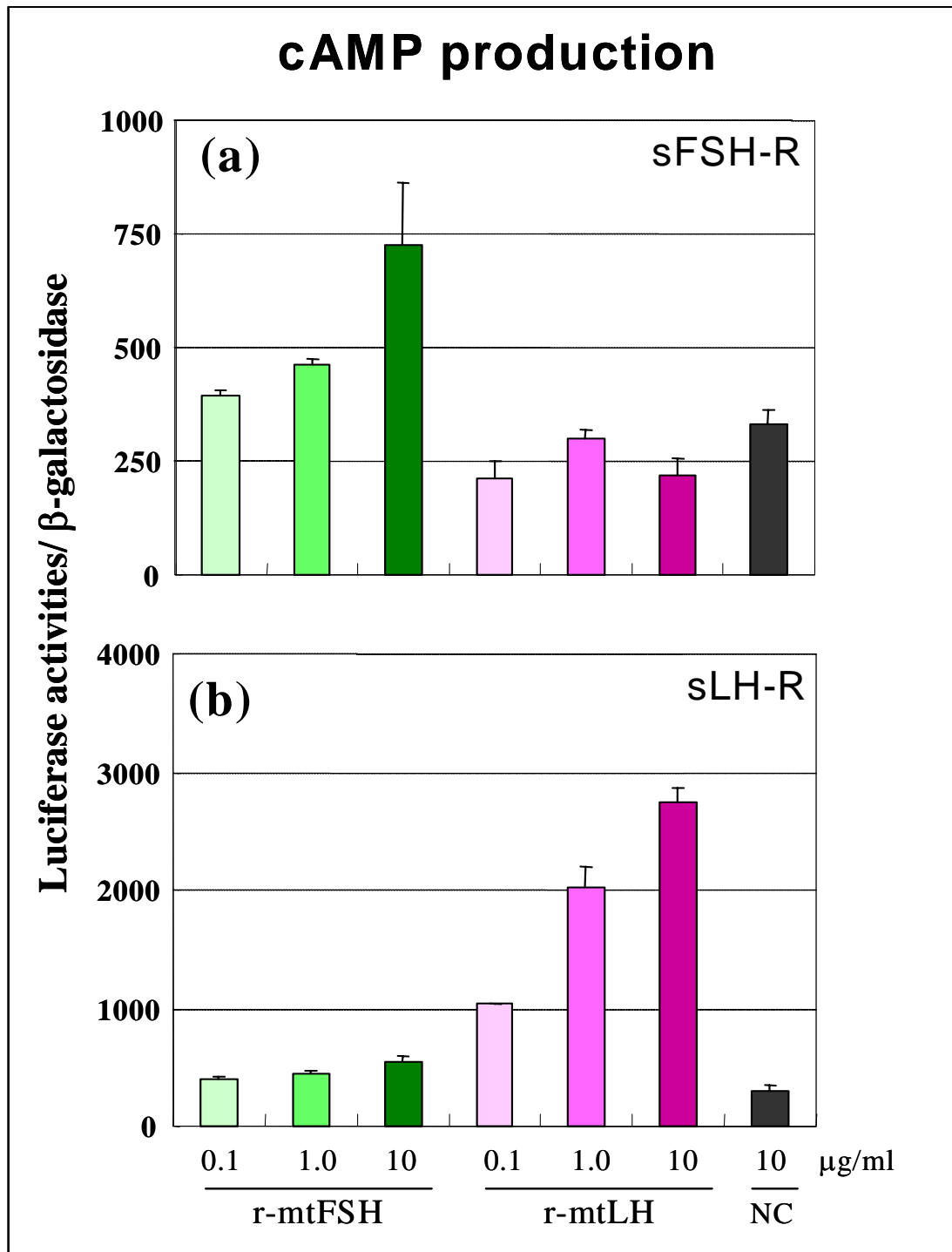


Fig. 32. Indirect measurement of r-mtFSH and r-mtLH bioactivities purified from silkworm. cAMP responsive luciferase reporter and amago salmon gonadotropin receptor constructs (FSH receptor (a); LH receptor (b)) were transiently transfected into COS-7 cells and r-mtFSH or r-mtLH was added as indicated. After 8 h, luciferase activities were measured.

나. 직접법 (Enzyme-immunoassay System, EIA)

(1) 도입 세포 : COS-7 cell

(2) 도입 방법 : CaCl₂

(3) receptor : amago salmon FSH/LH-수용체

(4) 실험 방법

24well plate에 세포 계대.

1일 배양(CO₂ incubator(5%), 37°C)

transfection	pCRE-luc (200ng/ μ l)	1.0 μ l
	β -gal(pcDNA3) (200ng/ μ l)	1.0 μ l
	sFSH/LH-R (200ng/ μ l)	1.0 μ l
	2.5M CaCl ₂	2.5 μ l
	DDW	20.5 μ l
	2X BBS	25.0 μ l
	total	50.0 μ l

vortex.

spin down.

RT 20min.

각 well에 50 μ l씩 첨가.

18hr 후 배지 교환.

transfection 3일 후 DMEM배지 원액으로 washing.

배지 250 μ l 첨가

호르몬 처리.(10⁻¹, 1, 10 μ g/ml)

CO₂ incubator(5%), 37°C 30min.

배지 제거.

lysis reagent 1B 200 μ l/well

RT. shaking 10min.

sample 회수 후 cAMP 농도 측정.

(5) cAMP assay

Biotrak Enzymeimmunoassay(EIA) System(Amersham Biosciences, UK)

-96well plate에 standard 와 sample을 분주

	1	2	3	4	5	6	7	8	9	10	11	12
A	B	B	400	400	S	S	S	S	S	S	S	S
B	NSB	NSB	800	800	S	S	S	S	S	S	S	S
C	0	0	1600	1600	S	S	S	S	S	S	S	S
D	12.5	12.5	3200	3200	S	S	S	S	S	S	S	S
E	25	25	S	S	S	S	S	S	S	S	S	S
F	50	50	S	S	S	S	S	S	S	S	S	S
G	100	100	S	S	S	S	S	S	S	S	S	S
H	200	200	S	S	S	S	S	S	S	S	S	S

(B : substrate blank, NSB : non-specific binding, s : sample, 0~3200 : standard)

-NSB well에 lysis reagent 1B, 2B를 각각 100 μ l씩 첨가.

-zero standard(0) well에 lysis reagent 1B 100 μ l씩 첨가.

-standard well에 각각의 standard 용액을 100 μ l씩 첨가.

-sample well에 sample 100 μ l씩 첨가.

-blank와 NSB well을 제외한 모든 well에 antiserum 100 μ l씩 첨가.

-3~5 $^{\circ}$ C, shaking incubator 2시간.

-blank를 제외한 모든 well에 cAMP-peroxidase conjugate를 50 μ l씩 첨가.

-3~5 $^{\circ}$ C, shaking incubator 1시간.

-well 안의 용액 모두 제거 후 wash buffer로 washing. (400 μ l씩 4회 반복.)

-TMB(enzyme substrate)를 모든 well에 150 μ l씩 첨가.

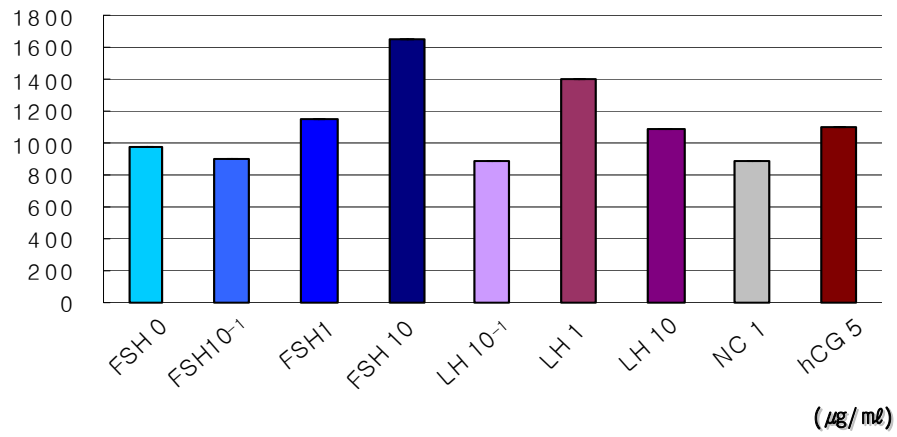
-15~30 $^{\circ}$ C (RT), shaking 1시간.

-파란색으로 변한 것을 확인한 후 1M sulfuric acid를 각 well에 100 μ l씩 첨가.

-30분 내에 450nm으로 OD측정.

sFSH-R (n=3)

sFSH-R	cAMP(fmol/well)	SD	%B/B ₀
F0	977.42	0.12	46.09
F10 ⁻¹	902.14	0.26	47.6
F1	1154.92	0.08	42.53
F10	1656.00	0.14	32.48
L10 ⁻¹	887.18	0.13	47.9
L1	1399.23	0.17	37.63
L10	1090.10	0.11	43.83
NC	893.16	0.07	47.78
HCG	1103.57	0.16	43.56



sLH-R (n=3)

sLH-R	cAMP(fmol/well)	SD	%B/B ₀
L0	1195.81	0.02	41.71
F10 ⁻¹	1287.55	0.09	39.87
F1	1262.62	0.16	40.37
F10	1258.13	0.17	40.46
L10 ⁻¹	1617.11	0.06	33.26
L1	2423.83	0.05	17.08
L10	2794.79	0.01	9.64
NC	1278.07	0.12	40.06
HCG	1949.67	0.05	26.59

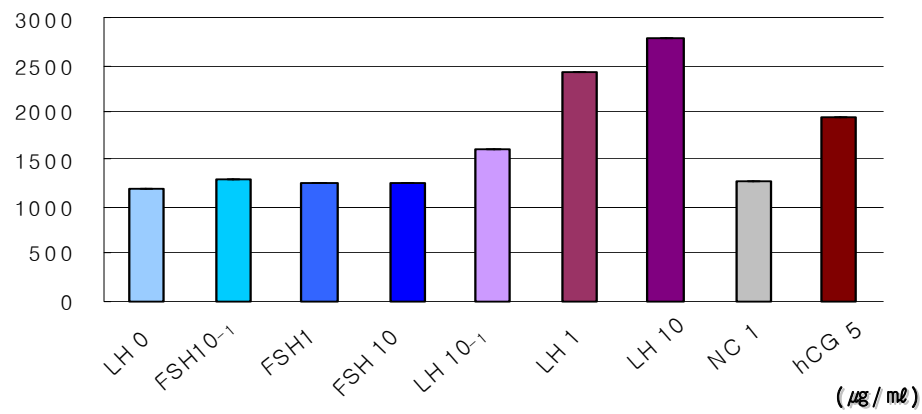


Fig. 33. Direct measurement of cAMP concentrations by r-mtFSH and r-mtLH treatments in COS-7 cells.

아래 Fig. 33에서는 세포내의 cAMP 농도를 직접 측정된 결과 역시 Fig. 32에서 보이는 간접적인 리포터어세이 방법과 유사하였음 (Fig. 33).

다. 스테로이드호르몬 측정법 (Radio-immunoassay System, RIA)

- 무지개송어 4년생 암컷 해부 (무게 5.6 kg; 생식소 중량지수, 17.9%)
- 난모세포를 Hank's buffer 세척 - 성숙난모세포의 배양



- FSH 및 LH의 투여 (농도: 13-1300 ng/ml)
- 1일간 배양 후 배지로 방출된 스테로이드호르몬의 측정

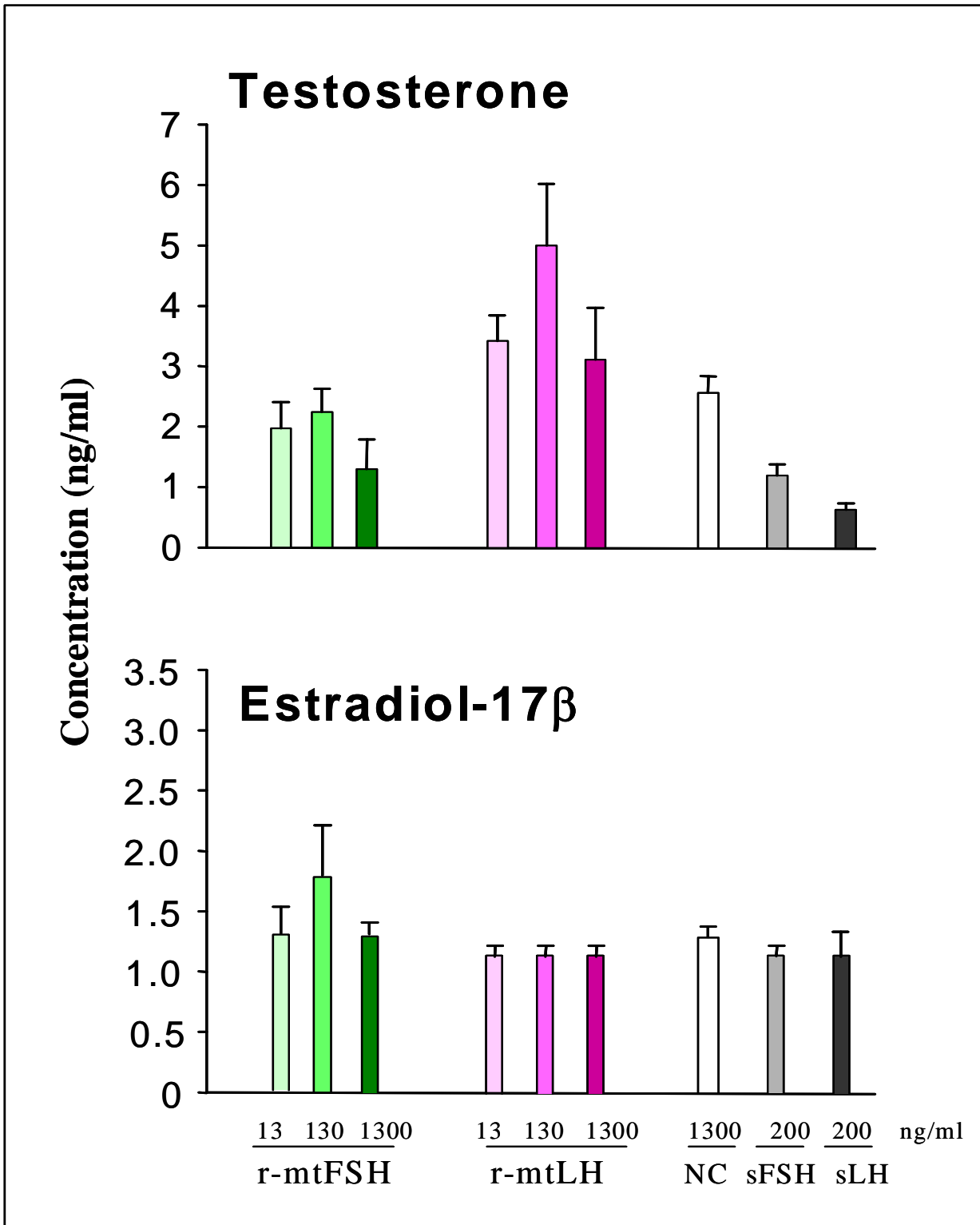


Fig. 34. Bioactivities of r-mtFSH and r-mtLH on steroid hormone synthesis in mature rainbow trout oocytes *in vitro*.

2. *In vivo* bioassay

가. 실험 재료 : 성숙초기의 무지개송어 (평균체중 200 g) 20마리

나. Procedure

- 무지개 송어 무 작위적으로 분리 수용 후 수조에 적응.
- 대조군 혈액 채취(0hr), 호르몬 복강 내 주사.
- 3개 그룹의 개체들의 혈액 채취 (18,24,72hr). 조직 해부, 조직 고정 (Bouin's액).

다. 실험 내용

- 주사 전 실험군 : 5마리
- 그룹 1 대조군 : 5마리(PBS/BSA/silkworm hemolymph)
- 그룹 2 mtFSH투여군 : 5마리(20 μ g/200g BW(PBS/BSA))
- 그룹 3 mtLH투여군 : 5마리(20 μ g/200g BW(PBS/BSA))

라. 채취한 혈장으로부터 스테로이드 호르몬의 측정.

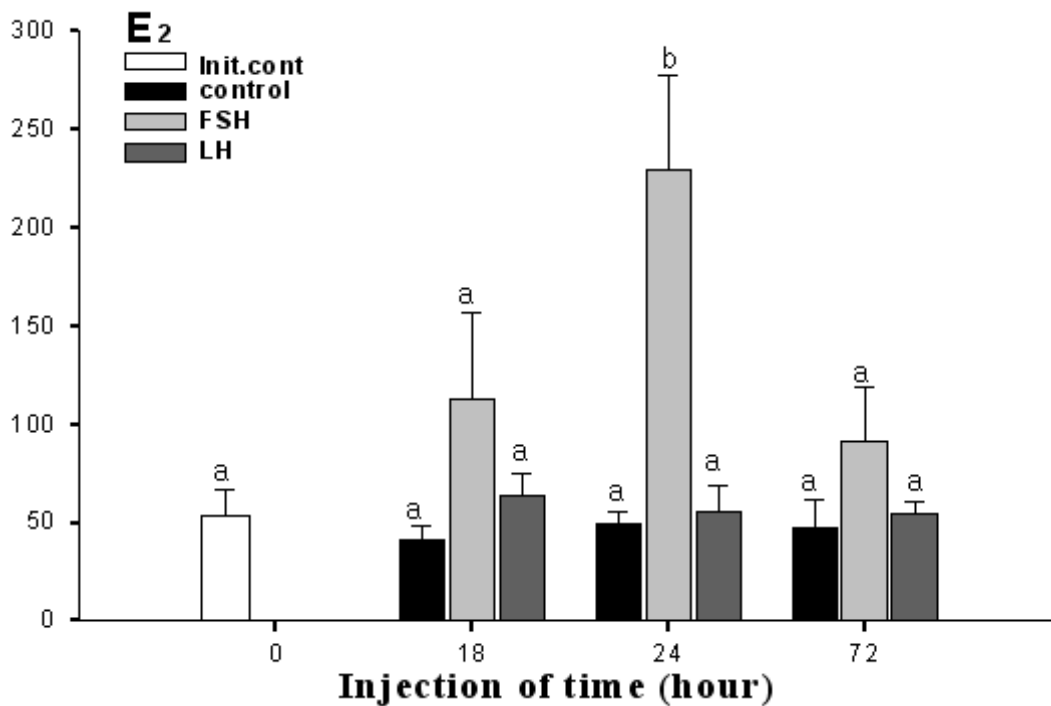


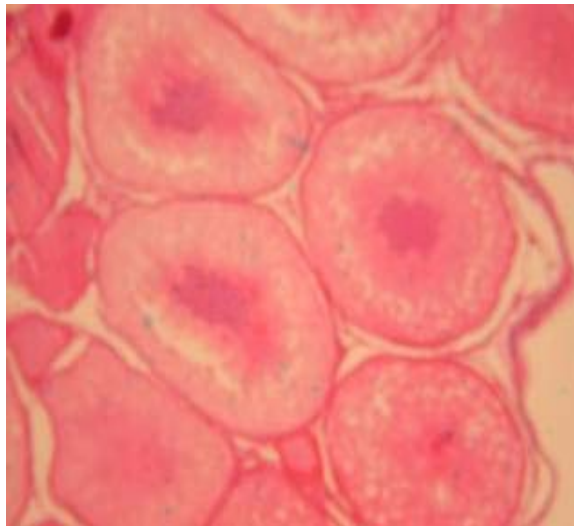
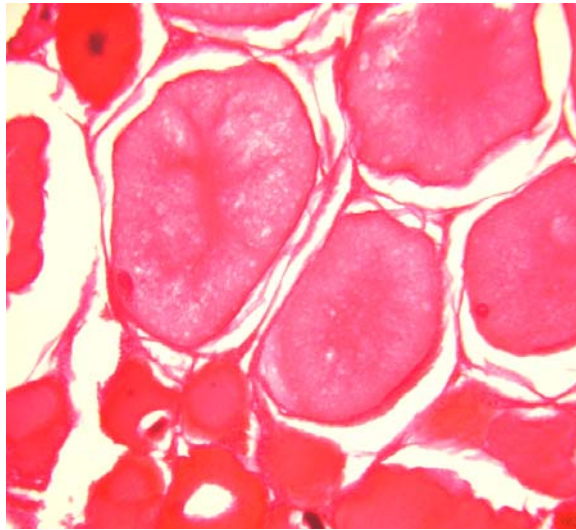
Fig. 35.measurement of steroid hormone in plasma.(RIA)

마. 난모세포의 조직학적 관찰

- 정제된 유전자재조합단백질의 생체 내 기능을 확인하고 궁극적으로 열목어의 번식 제어에 필요한 기본적인 데이터를 확보하기 위하여 열목어와 같은 연어과어류인 성숙초기의 무지개송어 (평균체중 200 g) 20마리에 유전자재조합 FSH, LH를 복강 내 주사하여 3일 후 생식소의 발달정도를 조직학적으로 관찰하였다.

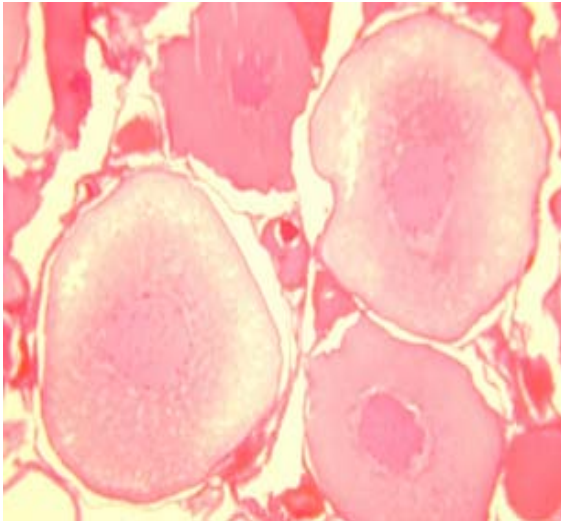
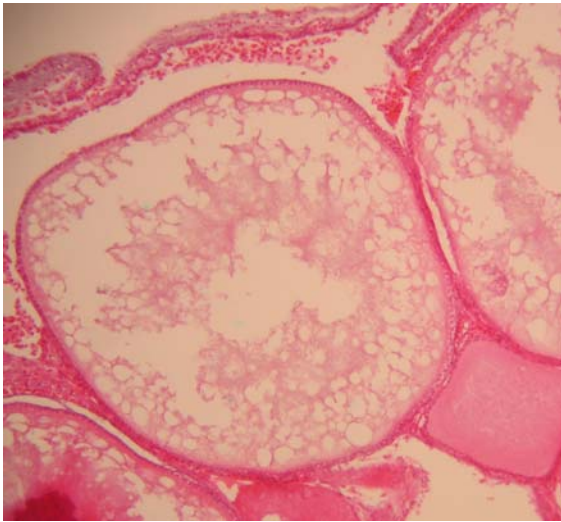
- 보우인액고정 ⇒ 헤마톡실린/에오진 염색법 ⇒ 검경

(A) Hemolymph-injected control fish



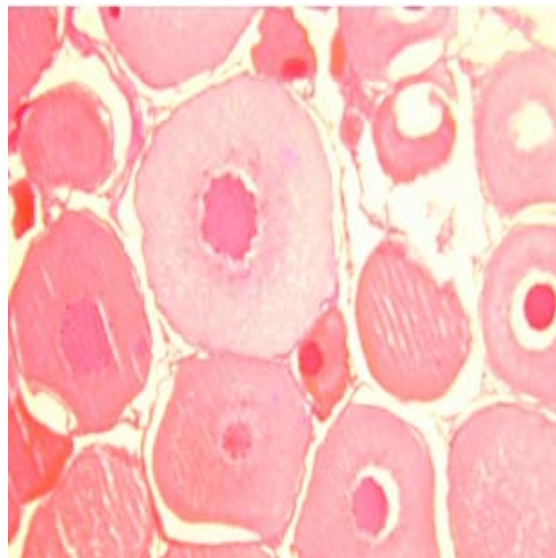
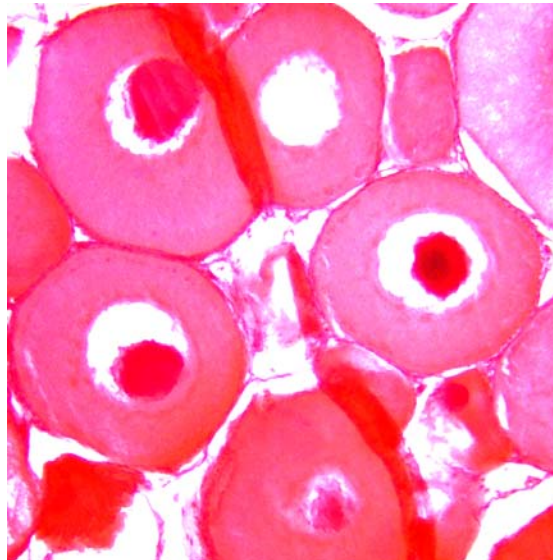
250 μm (X 100)

(B) mtFSH injected fish



250 μ m (X 100)

(C) mtLH injected fish



250 μ m(X 100)

Fig. 36. Hematoxyline-eosin staining of ovary in sexually early recrudescence rainbow trout. (A) Hemolymph-injected control fish, (B) FSH-injected fish, (C) LH-injected fish. $\times 100$.

- 열목어와 같은 연어과어류인 성숙초기의 무지개송어 (평균체중 200 g)의 복강에 유전자재조합 FSH 또는 LH를 1회 20 μ g 주사한 실험결과
 - 1) 대조군에 비하여 FSH를 주사한 무지개송어의 난모세포가 현저히 발달됨.
 - 2) 난황의 흡수가 증가하여 세포질 내에 난황이 축적된 과립구의 형성이 촉진됨.
 - 3) 난황형성기의 증기에 도달한 난모세포가 다수 출현함.

제 4 장 연구 개발 목표 달성도 및 대외 기여도

1절. 목표 달성도

1. 열목어의 생식선 자극 호르몬 2종류 (FSH, LH)의 유전자 재조합을 완료하였고, 생체 지속성을 증가시킬 수 있는 당 수식 부위가 삽입된 도입체 구성을 완성함.
2. 유전자 재조합 단백질을 외부 세포에서 대량 생산하고 추출 및 정제하는 방법을 확립하였음.
3. 생물학적 활성을 시험관 및 생체 내 실험을 통하여 확인하였으며, 생산된 FSH 및 LH 호르몬은 각각의 수용체 특이적인 반응이 나타났음.
4. 학술회의 (국제, 국내 포함) 발표: 3년간 10건
 - 가. 열목어(*Brachymystax lenok*)의 생식선 자극 호르몬 대량 생산을 위한 기초 연구. (2003년도 수산 관련 학회 공동 학술 대회, 2003-05-16)
 - 나. Molecular cloning of Gonadotropin Subunit cDNAs in the Manchurian Trout(*Brachymystax lenok*). (7th International Symposium on Reproductive Physiology of Fish, 2003-05-19)
 - 다. 열목어 생식선 자극 호르몬의 cDNA cloning 및 CHO세포를 이용한 발현검토. (2003년 한국양식학회 추계 학술 발표 대회, 2003-10-24)
 - 라. Biotechnological rescue for an endangered fish species. (Korea conference on innovative science and technology, 2003-11-13)
 - 마. A rescue strategy for endangered fish species. (2003년 생명과학 국제 심포지움, 2003-12-05)
 - 바. Recombinant manchurian trout *Brachymystax lenok tsinlingensis* FSH, LH and TSH production in small scale system using CHO cells. (Aquaculture, 2004-03-03)
 - 사. 포유동물 유래의 세포를 이용한 열목어 유전자 재조합형 생식선 자극 호르몬(LH)의 생산. (2004년도 수산 관련 학회 공동 학술 대회, 2004-05-14)
 - 아. Nucleotide sequence and production of Manchurian trout (*Brachymystax lenok*) luteinizing hormone. (5th International Symposium on Fish Endocrinology, 2004-09-06)
 - 자. 유전자 재조합형 어류 생식선 자극 호르몬의 생산과 활성. (2005년 한국양식학회 춘계 학술 발표 대회, 2005-05-20)
 - 차. Production of recombinant Manchurian trout gonadotropins. (15th International Congress of Comparative Endocrinology, 2005-05-25)
5. 국제 학회지 논문 발표: 3년간 SCI 2편, SCI 1편 준비 중
 - 가. Gonadotropin in the Manchurian trout, *Brachymystax lenok tsinlingensis*. (Fish Physiol. Biochem., 28, 89-90, 2003)
 - 나. Expression of gonadotropin genes in the Manchurian trout *Brachymystax lenok* and production of recombinant gonadotropins. (Fisheries Science, 71, 1191-1199, 2005, in press)
 - 다. Biological activities of recombinant Manchurian trout gonadotropins. (in preparation)

2절. 기대 효과

1. 경제적 측면 (성과의 산업계 이전 및 산업화를 통한 기대효과 등)

- 가. 수산물의 남획으로 인한 멸종 위기에 있는 해양 생물의 번식을 보존 유지 개발함으로써 국내 수산업에 큰 이익을 가져다 줄 것임.
- 나. 향후 발생되어지는 새로운 유전학적인 기초 지식을 축적할 수 있음.

2. 학술적 측면 (성과의 타 산업 및 학문에 미치는 효과 등)

- 가. 수산 생물의 유전학적인 연구 능력과 분석력이 증진됨.
- 나. 자연 자원 조성을 위한 과학적이고 합리적인 방법 개발.
- 다. 어종 고유의 호르몬과 동일한 물질을 대량 확보 가능.
- 라. 적절한 번식 시기에 투여하는 인공 번식 방법의 이용 가능.

제 5 장 연구 개발 결과의 활용 계획

1. 열목어의 친어 관리/종묘 생산에 유용하게 사용될 수 있는 유전자재조합 단백질의 혈액 내 잔존성을 방사면역측정법으로 조사할 예정이다.
2. 연어과 어류의 성숙 개체에서 난모 세포 발달을 촉진시키는 호르몬의 영향을 생체 내 실험으로 확인할 계획임.
3. 정원세포 및 정소의 생식 세포 발달에 미치는 호르몬의 영향을 시험관 및 생체 내 실험을 통하여 조사할 필요성이 있음.
4. 연어과 이외의 다른 어종 (잉어과 어류, 뱀장어 등)에서 호르몬의 생물학적 활성을 조사하여 타어종의 종묘 생산에 활용될 수 있는지 조사할 계획임.

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