최 종
연구보고서

수산 동·식물로부터 유전자 조작에 의한 천연단백분해효소 저해제 (protease inhibitor)의 대량생산 및 수산제품 응용기술개발

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본 보고서를 “수산 동·식물로부터 유전자 조작에 의한 천연 단백분해효소 저해제(protease inhibitor)의 대량생산 및 수산제품 응용기술개발” 과제의 최종보고서로 제출합니다.

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요 약 문

Ⅰ. 제 목

수산 동·식물로부터 유전자 조작에 의한 천연단백분해효소저해제(protease inhibitor)의 대량생산 및 수산제품 응용기술개발

Ⅱ. 연구개발 사업의 목적 및 중요성

1. 연구개발 사업의 목적

최근 국민소득의 증대에 따라 국내에서는 고소득사회에 따른 건강요구형 식품의 수요가 날로 급증할 것으로 예상된다. 또한 축육을 주식으로 하는 서양식 식생활은 여러 가지 성인병 및 암을 유발할 뿐 아니라 최근에는 광우병 등의 영향을 소비자들이 기피하고 있는 실정이다. 따라서 상대적으로 수산식품의 소비가 급격하게 증가함에 따라 유통기간 연장 등 수산식품에 관한 관심이 증가하고 있다. 수산식품 중 맛살류(어묵), 젓갈류의 경우 일정한 유통기간을 가질 수밖에 없는데, 이것은 수산물의 근육이나 조직 자체에 함유되어 있는 내인성 효소 및 미생물이 분비하는 외인성 효소의 작용이 매우 중요한 작용을 하는 것으로 연구되었다 특히 맛살류나 젓갈류의 주원료는 어류이므로 단백분해효소의 작용이 매우 중요하다. 단백분해효소는 surimi 조직에 작용하여 고유의 풍미나 독특한 조직감이 사라지는 등 품질열화가 일어나게 한다. 따라서 이들 제품의 유통기간 연장 및 품질 향상을 위해서는 효과적으로 단백분해효소의 활성을 억제하는 것이 필수적이다. 그러나 현재 농·축산물 유래 천연 단백분해효소 저해제에 대한 연구에 비하여 수산물 유래 저해제에 대한 연구는 초보단계에 머물러져 있으며, 수산물 유래 단백분해효소 저해제의 대량생산에 대한 연구는 아직 시작단계에 머물러져 있는 실정이다. 또한 생물공학기술을 통한 수산물 유래 단백분해효소 저해제의 생산기술은 아직 개발단계에 있으며, 대부분의 저해제의 경우 아직 유전자의 동정조차 이루어지지 않고 있는 실정이다.

또한 천연효소 저해제가 첨가된 맛살제품의 품질변화 분석 및 저장기간 예측에 대한 연구는 거의 없다. 즉 저장조건이 포장 맛살 등 수산연제품 조직의 단백분해효소 활성과 효소-저해제간의 반응활성에 미치는 영향은 거의 보고된 바가 없다. 따라서 어란(연어, 청어, 물곰치, 빙어, 명태 알 등) 및 해조류(녹조, 홍조 등) 유래 수산 동·식물로부터 유전자 조작을 통하여 천연단백분해효소 저해제를 대량생산하는 기술을 개발하고, 맛살류 등 수산식품의 품질의 고급화 및 유통기간을 연장을 도모

함으로써 소비확대 및 수출 증대 등 수산업 발전에 기여하는 것이 본 연구의 목적이다.

2. 중요성

수산식품의 품질 열화는 크게 미생물 대사에 의한 부패, 화학반응에 의한 변패, 내인성 및 외인성 효소반응에 의한 물성변화 및 변패로 나누어진다.

내인성 및 외인성 단백분해효소에 의한 품질 열화는 수산식품 유통기간의 결정에 매우 중요하다. 맛살류의 경우 가열공정 동안 내온성 단백분해효소에 의해 단백질이 분해되어 독특한 조직감을 구성하는 gel 구조가 파괴되며, 젓갈류는 자가소화효소 및 미생물이 분비하는 단백분해효소에 의해 어느 정도 기간이 지나면 과숙성되어 고유의 풍미가 사라지는 등 품질열화가 일어난다. 따라서 이들 제품의 유통기간 연장 및 품질향상을 위해서는 효과적으로 단백분해효소의 활성을 억제하는 것이 필수적이다. 단백분해효소의 활성을 억제하기 위하여 여러 방법들이 적용되어왔으나 천연저해제를 이용하는 것이 바람직한 방법의 하나로 알려지고 있다. 그러나 현재 맛살류의 gel 파괴를 방지하기 위하여 연구되고 있는 천연 단백분해효소 저해제의 대분분은 대두를 비롯한 쌀, 감자, 수수, 소와 돼지의 혈장, 우유, 달걀흰자 등 농·축산물에서 추출되며 수산식품에 존재하는 단백분해효소에 완벽하게 적용되지 않는다. 현재 농·축산물 유래 천연단백분해효소 저해제에 대한 연구에 비하여 수산물 유래 저해제에 대한 연구는 미비한 실정이며, 수산물 유래 단백분해효소 저해제의 대량생산에 대한 연구는 시작단계에 머물러 있다. 그리고 천연 효소 저해제가 첨가된 포장 맛살 제품의 품질 변화 분석 및 저장기간 예측에 대한 연구는 거의 없는 실정이다. 수산식품에는 수산물 유래 천연단백분해효소 저해제의 이용이 가장 효과적이라고 생각되며 수산식품의 유통기간 연장과 품질향상을 위해서는 수산물 유래 단백분해효소들의 활성억제에 효과적인 천연 저해제를 개발하는 것이 시급하다.

또한 수산물 유래의 천연 단백분해효소 저해제의 적용은 맛살 등 전통수산식품의 품질과 안정성을 강화하여 외국 식품의 수입억제 및 국내 수산식품의 해외 진출 등을 도모할 수 있으며 수산물 유래 천연 단백분해효소 저해제의 새로운 시장을 개척하여 관련 산업의 발전을 가져올 뿐만 아니라 우리문화의 우수성도 함께 밝혀내는 매개체 역할을 할 것이다. 또한 전통 수산식품을 통한 국민 건강 증진 및 식문화의 보존에도 보탬이 될 것으로 본다.

Ⅲ. 연구개발사업의 내용 및 범위

1. 천연 단백분해효소 저해제의 대량생산 및 수산식품에의 응용 기술 개발

가. 수산동물 (명태, 도치, 도루묵, 물곰치, 빙어, 연어) 알의 단백분해효소저해제의 검색

수산동물의 알을 이용하여 단백분해효소를 column chromatography로 분석하여 분리•정제하여 단백분해효소 저해활성 측정 방법을 확립 (Papain activity 저해 활성측정)하고 HPLC, SDS-electrophoresis를 행하여 단백분해효소 저해제의 순도 및 분자량 측정을 확립하였다.

나. 수산동물에 함유된 천연 단백분해효소저해제의 분리 및 정제

단백분해효소저해제 정제 및 특성실험을 하였으며, 저해효과 및 저해기작 분석하였다. 합성 및 천연 물곰치알 단백분해효소저해제의 효과 및 저해기작 실험을 하였으며, Cathepsin 및 trypsin에 대한 물곰치알 저해제의 저해활성을 비교분석하였다. 상업용 제품(Egg white 및 potato inhibitor)과의 비교 실험도 병행하였다.

다. 분리된 천연 효소저해제의 효과 및 저해기작 평가

Ki and Vmax value 측정에 의한 저해기작을 평가하였고, 온도, pH 및 수분활성의 영향 분석하였다.

라. 효과가 우수한 수산물 유래 저해제의 아미노산배열 분석, 유전자 동정 및 PCR을 통한 유전자 복제

물곰치 저분자 단백분해효소저해제의 아미노산배열 분석, 유전자 동정 및 PCR을 통한 유전자 복제실험을 하였으며, *E. coli* 균에 대한 cloning을 하였다. Cloned *E. coli* 균으로부터 재조합 단백분해효소저해제의 최적 배양조건을 확립하여 재조합 단백분해효소저해제의 정제 및 활성 비교 실험을 하였으며, Calpain inhibitor의 저해활성 비교실험도 행하였다.

마. 연어알 단백분해효소 저해제의 재조합 효모의 발효공정 및 효소저해제 생산의 최적화

효모에 발현된 연어알 단백분해효소 저해제의 배지 조성 및 배양조건의 최적화를 확립하였다.

바. 재조합 미생물의 발효를 통한 천연 효소저해제의 대량생산공정과 분리 정제기술의 확립

연어알 단배분해효소 저해제의 정제 방법 및 저해활성을 연구하였다.

사. 재조합 효소저해제가 함유된 수산식품의 품질향상

단백분해효소저해제를 첨가한 surimi를 제조하여 품질 특성 실험을 하였으며, 화장품 관련 효소에 대한 저해활성 실험을 하였다.

2. 단백분해효소 저해제의 대량 생산 및 생산 공정의 최적화

가. 해조류(홍조 및 녹조)로부터 단백분해효소 저해제를 검색

해조류 2종(홍조인 방사무늬 김, 녹조인 창자파래)으로부터 단백질분해효소 저해제를 확인하고 단백질분해효소 저해활성을 측정하였다.

나. 해조류에 함유된 단백분해효소저해제의 분리 및 정제

홍조류(*P. yezoensis* and *tenera*) 및 녹조류(*E. intestinalis*)의 단백분해효소저해제 효과의 비교 평가를 행하였으며, *P. yezoensis*로부터의 단백질분해 효소저해제 분리공정효율을 평가하였다.

다. 해조류 효소저해제의 효과 및 저해기작 평가

*P. yezoensis*의 단백분해효소저해제의 특성을 온도 및 산도(pH)에 따라 beef plasma protein, egg white, potato powder 등 기존의 농·축산물 유래 저해제와 비교 평가하였다.

라. 물곰치 단백분해효소 저해제의 N-terminal 분석 및 합성

물곰치 알로부터 유래되는 단백분해효소저해제의 N-terminal을 근거로 peptide 합성을 행하였다.

마. 연어알 단백분해효소저해제의 재조합 벡터 및 재조합 효모 개발

Cystatin 발현 벡터 pYES2/NT_C (cystatin)를 효모(*S. cerevisiae* YPH499)에 형질전환 하였으며, 재조합 벡터를 제작해 효모에서 발현하였다.

바. 물곰치알 재조합 벡터 및 재조합 효모 개발

재조합 벡터를 제작하여 물곰치 유래 단백분해효소저해제의 형질이 전환된 효모를 개발연구를 하였다.

Ⅳ. 연구개발결과

1. 수산동물 유래 단백분해효소 저해제의 개발 및 수산식품에의 응용

가. 명란(Alaska pollock egg)로부터 단백분해효소 저해제 정제 및 특성

명란 유래의 단백분해효소저해제는 ammonium sulfate precipitation, ion exchange , gel permeation, HPLC의 단계를 거쳐 정제 되었다. 단백분해효소의 경우 열변성 된 알에서는 정제가 잘 되지 않았고, 열변성이 없는 알에서 정제된 단백분해효소보다 저해활성이 낮게 나타났다. 단백분해효소의 정제 정도는 ammonium sulfate precipitation, ion exchange, gel permeation에서 22.7%, 15.3%, 4.4 %로 각각 나타났다. 명란의 단백분해효소저해제는 gel permeation chromatography 패턴에 의하면 66.7kDa과 16kDa의 분자량을 갖는 두 종류로 나타났다. 두 종류의 단백분해효소저해제의 경우 papain을 저해하는 cystein 단백분해효소저해제로 생각되어진다.

나. 물곰치알(Glassfish egg)로부터 저분자단백분해효소저해제 (Low-molecular wight protease inhibitor) 정제 및 구조 분석

물곰치알로부터 정제된 67과 18 kDa의 두 종류의 단백분해효소저해제는 affinity chromatography에 의해 정제되었다. 단백분해효소 저해제의 수율과 순도는 18 kDa의 분자량을 갖는 단백분해효소저해제의 경우 0.25%와 46.69 이었다. 18kDa의 아미노산배열은 N-his-ala-asn-arg-val-met-pro-glu-met-asn-met-glu-tyr-met-glu-ala-C 이었다. 물곰치알 유래의 단백분해효소는 papain에 비길항적 효소로 0.44 nM의 Ki값을 가졌다. 물곰치알 유래의 단백분해효소저해제는 50~65℃와 pH 8에서 안정적이며, egg white 단백분해효소저해제에 비해 cathepsin에 대한 저해활성이 높게 나타났으며, papain에 대한 저해활성은 그렇지 않았다. 따라서 물곰치알 유래의 단백분해효소저해제는 cysteine계 단백분해효소저해제 중 하나로 생각되어진다.

다. 물곰치알 (Glassfish egg)로부터 고분자단백분해효소저해제 (high-molecular weight protease inhibitor) 정제 및 구조 분석

Affinity chromatography와 electro-elution에 의해 67과 18 kDa의 두 종류의 물곰치알 유래의 단백분해효소저해제가 정제되었다. 이 중 고분자단백분해효소저해제(HMW)는 18.46 U.mg의 저해활성과, 0.07%의 수율과 131.86의 순도로 정제되었다. 물곰치 유래의 고분자단백분해효소저해제의 저해활성을 위한 최적의 온도와 pH는 각각 40 ℃, 6으로 나타났으며 5 ℃~50 ℃와 pH 5~6의 범위 내에서는 안정적이었다. 97.02 nM의 Ki를 갖는 papain과 비교하였을 때 길항적인 저해를 하는 것으로

보여 졌다. 또한 단백분해효소를 저해하는데 egg white 단백분해효소저해제보다 더욱 효과적이었다. 물곰치 유래의 고분자단백분해효소저해제는 family Ⅲ중 하나의 것으로 구분되어진다.

라. 연어알(salmon egg) 단백분해효소저해제의 정제 및 특성

연어알에서 ion exchange, gel permeation과 affinity chromatography로 정제된 단백분해효소저해제의 분자량은 72.6 kDa이다. 연어알 유래의 단백분해효소저해제는 1.50%의 수율과 8.344의 순도로 정제되었으며 SDS-PAGE의 결과로 보아 54.0과 18.6 kDa의 분자량을 갖는 두 개의 subunit으로 이루어져있다. 연어알 유래의 단배분해효소저해는 20~40 ℃와 약산(pH 6)에서 안정적이며 papain과 cathepsin을 저해하는 cysteine계 단백분해효소지만 chymotypsine은 아니다. 연어알 유래의 단백분해효소저해제는 cathepsin 저해활성은 egg white 단백분해효소저해제보가 효과적으로 나타나는 반면 papain에서는 그렇지 않은 것이 사실이다. 이러한 결과로 보아 연어알 유래의 저해제는 heterodimer이며, cysteine계 단백분해효소저해제로 구분되어진다.

마. 기타 어종 단백분해효소저해제 정제 및 특성

단백분해효소저해제는 청어, 연어, 빙어, 물곰치와 명태의 5가지 어종의 알을 이용하여 정제하였다 각 어종별 단백분해효소저해제의 분자량은 120, 89, 48.5, 17과 16.8 kDa으로 나타났으며 단백분해효소저해활성의 경우 청어나 명란보다 물곰치알 유래의 단백분해효조저해제가 가장 높았다. 물곰치 알 유래의 단백분해효소저해제의 단백분해효소 저해활성과. 순도는 19.70 U/mg과 164.70이며 50~65 ℃와 pH 8에서 비교적 안정적이다. 물곰치 알 유래의 단백분해효소저해제의 Ki는 4.44 nM이었다.

바. 재조합 *E.coli*로부터 물곰치 단백분해효소저해제 정제

어육연제품과 어육의 품질열화는 cathepsin과 alkaline 단백분해효소 등과 같은 어류 근육단백분해효소를 저해하는 것에 의해 방지할 수 있다. 품질열화는 방지 할 수 있는 최선의 방법은 어류 근육 단백분해효소를 천연 저해제롤 사용하여 저해는 것이나 천연물에서의 저해제의 정제 수율이 매우 낮고 산업적 이용의 가능성이 매우 낮다. 식품산업과 생물공학산업을 위해 단백분해효소 저해제의 대량생산은 중요하다. 따라서 물곰치알 단백분해효소를 *E. coli*.에 클로닝하고 재조합 단백분해효소저해제를 정제하여 천연단백분해효소저해제와 비교하였다. 물곰치알 유래의 단백분해효소가 19.70 U/mg의 저해활성을 갖는 것에 비해 재조합 단백분해효소 저해제의 저해활성은 7.117 U/mg 낮게 나타났다. 재조합 물곰치 단백분해효소 저해제의

amino acid sequence 중 Met과 Asn이 변화한 것이 저해활성을 감소시키는데 영향을 끼친 것으로 추론된다.

사. 재조합 효모의 연어알 단백분해효소저해제의 특성

pYES2/NT_C. SC medium uracil이 합성된 *Saccharomyces cerevisiae* YPH 499에 발현된 재조합 연어알 단백분해효소저해제는 재조합 효모의 선택배지에 배양하였다. 재조합 효모의 연어알 단백분해효소저해제의 생산은 pH 5.5에서 12시간동안 배양하는 것이 최적 조건이다. 배양 후 재조합 효모는 제거 하고, 재조합 연어알 단백분해효소저해제는 His-select nichel affinity chromatography에 정제하였을 때 61%의 수율과 5.60의 순도를 나타냈다. 재조합 단백분해효소저해제의 분자량은 35 kDa으로 papain과 cathepsin L과 비교하였을 때 높은 저해활성을 보였고 온도와 약산에 비교적 안정적이었다.

아. 천연효소저해제가 함유된 수산식품의 품질 향상 연구

*Saccharomyces cerevisia*e에 발현된 재조합 연어 단백분해효소저해제는 His-select nichel affinity chromatography에 의해 정제하였다. papain과 cathepsin과 비교하였을 때 재조합 연어 단백분해효소저해제의 저해활성은 7.45와 10.24 U/mg으로 각각 나타났으며 pH 5.0과 65 ℃까지의 온도 범위에서 안정적이었다. 재조합 연어알 단백분해효소저해제는 surimi-gel의 weakening을 저해하였으며 TCA-soluble peptide의 분해에 의한 Surimi의 분해와 비교하였을 때 재조합 연어 단백분해효소저해제를 100 μg/g이 되게 첨가하였을 때 가장 높은 저해활성을 가졌다. 재조합 연어 단백분해효소의 첨가량을 증가하게 되면 modori gel의 breaking force와 deformation이 현저하게 증가하였다($P<0.05$). 재조합단백분해효소저해제의 첨가는 drip의 생성을 적게 하는 동시에 surimi-gel의 미백을 증가시켰으며 더 많은 Myosin heavy chain을 보유하게 하였다. 재조합 연어알 단백분해효소저해제는 명태 Surimi의 단백질 degradation을 방지하는 정도가 egg white보다 뛰어나다. 따라서 재조합 연어 단백분해효소저해제는 surimi-gel의 품질 열화를 방지하기 위해 산업적으로 응용될 수 있을 것으로 생각되어진다.

자. 화장품 및 젓갈 관련 단백분해효소저해활성 비교

재조합 cystatin의 경우 elastase 저해활성 및 tyrosinase 저해활성은 나타나지 않았으나 collagenase 저해활성은 0.05 mg/mL의 농도에서 나타났다. 그러나 재조합 cystatin의 농도를 0.2 mg/mL까지 증가시켜 측정한 결과 저해활성정도가 확연히 차이가 나지 않았다.

차. 송어 혈장 단백질의 특성

송어혈장단백질을 surimi gel에 1 mg/g이 되도록 첨가하였을 때 무첨가구에 비하여 breaking force가 두 배 이상 증가하였다. 0.75 mg/g의 농도로 첨가하였을 때 가장 높은 값을 얻었으며 egg white를 2 mg/g이 되게 첨가한 것보다 breaking force가 높게 나타났다. 또한 송어혈장단백질을 첨가한 것은 surimi-gel의 미백효과가 나타났으며 수분의 함량이 감소하였다. 미백효과역시 0.75 mg/g에서 가장 높았으며, 그 이상의 농도에서는 오히려 미백정도가 감소하는 것으로 나타났다. 미백효과 역시 egg white를 2 mg/g이 되게 첨가한 것보다 뛰어났다.

송어혈장단백질을 0.75 mg/g을 첨가한 것은 myosin heavy chain(MHC)의 degradation을 저해할 수 있고, 그 이상 첨가한 것은 MHC의 degradation을 증가시킬 수 있다.

2. 단백분해효소저해제의 대량생산 및 생산공정의 최적화

가. 단백질함량비율이 높으며 손쉽게 구할 수 있는 김(홍조류)과 파래(녹조류)에서 단백질 성분 추출 및 단백분해효소저해제의 활성 연구

1) 김유래 단백질 분해효소 저해제

방사무늬 김의 단백질 성분은 ethanol추출법과 물과 sonication을 이용한 기계적 추출법을 이용하여 추출하고 이온교환크로마토그래피를 사용하여 정제하였다.

Ethanol로 추출한 방사무늬 김 유래의 단백질 성분은 ammonium silfate의 농도에 따라 그 양이 다르게 나타났으며, 0-20%의 ammonium sulfate에서 단백질의 양이 45 mg으로 다른 구간에 비해 월등히 높았으나 저해활성은 0.04 unit/mg로 타 구간에 비해 매우 낮게 나타났다. papain 저해활성은 40~60% 구간에서 16 unit/mg으로 가장 높게 나타났다. 또한 기계적 추출방법으로 추출한 단백질분해효소저해제는 ethanol 추출의 경우에 비하여 단백질이 전 구간에 걸쳐 고르게 추출되었고, papain 저해활성은 60~80%, 80~100%에서는 나타나지 않았고 나머지 구간에서는 ethanol 추출물과 유사하게 나타났다. 정제된 단백질분해효소저해제의 가장 높은 저해활성은 약 0.09 unit/mg이었고 상대적으로 (+) charge를 많이 함유한 단백질이 papain 저해하는데 특정한 역할을 한다는 것을 추론할 수 있다.

2) 파래 유래 단백질분해효소 저해제

파래의 경우 김의 ethanol 추출물과는 매우 다른 양상을 보였는데, 단백질은 ammonium sulfate의 농도가 0~20%, 20~40%의 구간에서 약 12 mg/mL로 다량 검출되었고 나머지 구간의 경우 0.5 mg이하의 미량만 검출되었다. 그러나 단백질의

양과는 상관없이 papain 저해활성은 0.1 unit/mg로 매우 낮았으며 80~100%구간에서 21 unit/mg의 저해활성을 갖는 것으로 나타났다. 따라서 80~100% 구간만 이온크로마토그래피를 수행한 결과 2개의 peak으로 구분되었으며 작은 단백질 peak의 저해활성이 상대적으로 높게 나타났다.

나. 해조추출물의 단백분해효소 저해효과 및 해조유래 저해제와의 특성비교

방사무늬 김에서 ethanol추출법과 기계적 추출법에 의해 추출하고 이온교환크로마토그래피로 정제한 단백분해효소저해제는 66 kDa보다 약간 큰 부근에서 관찰되었으며 이는 일반적으로 알려진 cystatin의 분자량 약 13 kDa보다 훨씬 크다. 본 실험결과에서는 방사무늬 김의 ethanol 추출물에 존재하는 단백질 분해효소 저해제는 (+) charge를 다량 함유하고, 분자량이 큰 것으로 보아 일반적으로 egg white cytatin보다는 훨씬 큰 단백질이라고 예상한다.

창자파래에서 단백질분해효소저해제의 추출은 김과 동일한 방법을 이용하였으며 단백질의 종류와 크기를 알아보기 위하여 전기영동을 수행하고 있다.

해조 유래 단백분해효소저해제는 ammonium sulfate의 농도, 온도, pH에 의해 저해정도에 차이가 나타났으며 40~60%구간의 저해제가 비교적 모든 온도에서 높게 나타났고, pH 6.0에서 가장 높은 저해활성을 가졌다. 해조 유래의 단백분해효소저해제는 25~37 ℃, pH 4.0 ~ pH 6.0에서 안정적이지만, 농, 축산물 유래의 단백분해효소저해제에 비해 낮은 온도에서 저해활성이 높게 나타났다.

다. *S. cerevisiae*에 의한 재조합 연어 cystatin 생산을 위한 대량발효와 배양공정의 최적화

재조합 연어 단백분해효소저해제의 생산을 위한 *S. cerevisiae* YPH 499의 배양과 성장 조건의 최적조건을 설정하기 위해서 shake flask에서 실험한 것을 기초로 하였다. 반응 표면 분석(RSM) 방법을 응용하여 5가지의 변수, 즉 medium의 pH, 배양시간과 YNB, amino acid, adenine의 양을 설정하여 실험한 결과, pH 5.70에서 6.68시간동안 배양하고, 2% galactose가 5.6 g/L가 되도록 첨가한 것이 최적 조건이었다. 최적 조건을 바탕으로 재조합 효모는 14 L의 발효조에서 350 rpm으로 진탕하면서 1.0 vvm으로 aeration해 준 결과 0.56 U/mL의 높은 수율을 얻었다. 또한 45%의 cold alcohol을 첨가하면, 재조합 효모로부터 재조합 연어 단백분해효소저해제의 정제를 좀 더 쉽게 할 수 있었다.

Ⅴ. 연구개발 결과의 활용계획

1. 기대 효과

가. 기술적 측면

관능이나 영양분의 손실을 최소한으로 하며 수산식품의 품질열화에 관여하는 내인성 및 외인성 단백분해효소의 활성을 억제하기 위한 방법으로 효소 저해제 첨가 및 열처리공정 개선 등이 연구되고 있다. 맛살류의 경우 gelation을 얻기 위하여 가열공정을 거치나 잔존하는 내열성 단백분해효소의 작용이 활발하므로 주로 달걀흰자 또는 감자에서 생산되는 단백분해효소 저해제를 첨가하여 품질열화를 억제한다. 비가열처리 식품인 젓갈류의 경우 숙성 후 미생물의 생육을 억제하여 외인성 효소의 활성을 억제하려는 연구가 있었으나 (김 등, 1999; 임 등, 2000), 효소 저해제의 사용에 대한 연구는 아직 체계적으로 이루어지지 않고 있다. 수산식품에는 어란, 말미잘, 해조류 등 수산동·식물에서 유래된 저해제를 사용하는 것이 가장 바람직하나 이에 대한 연구 및 유전자 조작을 이용한 단백분해효소 저해제의 대량생산 기술개발은 아직 초보단계에 머물러 있다. 그러므로 본 연구에서 수산동물에서 정제한 천연 단백분해효소 저해제를 미생물에서 대량생산하여 맛살(surimi) 제품의 품질변화 분석을 한 결과는 생물공학적인 측면뿐만 아니라 효소저해제를 이용한 수산식품의 품질열화 연구의 기술적인 토대를 이루었다고 판단된다.

본 연구의 기술적인 측면을 요약하면

첫째, 수산물 (어란 및 해조류)에 존재하는 천연 단백분해효소 저해제에 대한 정보 및 기초 자료가 축적되었으며,

둘째, 유전자 조작 등에 의한 수산물 유래 천연 단백분해효소 저해제의 생물 공학적 대량생산기술이 확립되었으며, 특히, 수산물유래 단백분해효소저해제는 peptide의 일종(Mw 10-20 kDa)으로 이의 유전자 조작에 대한 연구는 아직 시작단계이다. 그러므로 수산물유래 단백분해효소저해제의 유전자를 함유한 재조합 미생물에 대한 기초자료 및 응용기술이 개발되었다.

셋째, 수산식품의 품질열화를 방지하여 저장기간을 증대하기 위한 천연 효소 저해제 이용 기술이 확립되었으며, 특히, 대표적인 수산식품인 맛살의 주요 품질열화 요인인 단백분해효소를 저해하여 수산식품 열화의 억제 및 이로 인한 저장(유통)기간의 확대가 예상된다.

넷째, 수산물 유래 천연 효소저해제에 대한 생화학, 생리학적 기작 및 분리, 정제기술 연구에 기여하였다고 판단된다.

나. 경제 · 산업적 측면

첫째, 천연 단백분해효소 저해제의 상품화가 이루어지면 수산 산업을 포함하여 최소 50억 이상의 신규 시장의 개척이 가능하며,

둘째, 품질열화 억제에 의한 맛살류의 고급화와 유통기간의 획기적 연장으로 인하여 국내 및 해외 판매량과 판매 이윤이 크게 증가할 것으로 예상되며,

셋째, 유전자공학, 단백질공학, 발효공학, 분리/정제, 효소학, 식품공학 등 다양한 학문, 기술 분야의 유기적인 연계활동을 촉진하여 우리 바이오산업의 활성화를 가져올 것으로 예상되며,

넷째, 천연 효소 저해제를 이용한 품질열화 방지기술의 파급으로 인한 농·축산 식품산업의 활성화가 기대되며,

다섯째, 수산자원으로부터 단백분해효소저해제가 성공리에 개발되어 어민들의 소득증대 및 관련 산업의 활성화가 이루어 질 것으로 본다.

2. 활용방안

첫째, 천연 단백분해효소 저해제의 상품화는 우선 참여기업에서 시행하도록 유도하며, 경쟁력 있는 새로운 바이오 벤처기업의 설립을 검토한다.

둘째, 맛살제품 유통 시 천연 효소 저해제를 첨가하여 획기적인 저장기간 연장을 꾀한다.

셋째, 수산식품 분야 뿐 아니라 농·축산식품 분야로 응용 범위를 확장한다.

넷째, 수산물 유래 천연 효소 저해제의 의약품으로서의 가능성을 검토 한다

SUMMARY

Surimi, a stabilized fish myofibrillar protein, is the primary ingredient in

surimi-based products. Gel functionality, such as texture and color, of surimi seafood is the most important aspect of product quality. However, autolysis by endogenous heat stable proteases causes an irreversible destruction of the surimi gel structure, especially at temperatures close to 60℃. This gel softening of surimi-based product is called as "modori phenomenon". Therefore, surimi industry have been using the commercial protease inhibitors to prevent modori (gel softening) phenomenon and to maximize the gel strength of surimi. The most commonly used inhibitors are bovine plasma protein (BPP), chicken egg white protein, potato powder, and whey protein concentrate. Because there is some side effect on surimi-based product such as change of color when these protease inhibitors were applied, fish protease inhibitor is thought to be the best one to prevent modori phenomenon.

The inhibitor of cysteine protease was first isolated from chicken egg white in 1968. This inhibitor was further characterized as cystatin classified as the first member of the cystatin superfamily. The cystatin superfamily is divided into three structurally related families; stefins, cystatins, and kininogens. Family 1 (stefin) lacks both disulfide bridges and carbohydrates. Stefin has a molecular mass of around 11 kDa, is the smallest in the cystatin superfamily. Family II (cystatin) is also single chain with one domain protein and about 2 kDa larger than the family I inhibitor. The polypeptide chains of cystatins contain two disulfide bonds near their C-terminus. Family III (kininogen) consists of a N-terminal heavy chain combined with a variable length light chain. The heavy chain has three cystatin-like domains. Based on the length of the light chain, the kininogen is divided into two sub-families; a high molecular weight kininogen (HMW kininogen, ~ 120 kDa) and a low molecular weight kininogen (LMW kininogen, ~ 68 kDa).

The interaction between proteases and their inhibitors was a target of intensive study for the last two decades. Protease inhibitors were purified from ovarian fluid carp, egg and muscle of chum salmon, muscle of white croaker, Atlantic salmon and Arctic charr, and hake, Argentine anchovy, castaneta, rough sead, and sea trout.

In this study, protease inhibitors with strong inhibitory activity against cysteine proteases causing the gel softening of surimi-based product were

screened from different fish eggs and seaweed and then determined their characteristics. Some protease inhibitors were cloned in microorganism for mass production, which was then applied to surimi-based product and compared with other commercial inhibitor.

Chapter Ⅰ. Mass production of natural protease inhibitor and its application for seafoods

Section 1. Characteristics of Protease Inhibitor Purified from the Eggs of Alaska pollock (*Theragra chalcogramma*)

Protease inhibitors were purified from the eggs of Alaska pollock (*Theragra chalcogramma*) by the purification steps of ammonium sulfate precipitation, ion exchange, gel permeation, and high performance liquid chromatographies (HPLC). The protease inhibitor was not purified well from the heated eggs of Alaska pollock. And it showed the lower specific inhibitory activity than the unheated eggs. The purification yields after ammonium sulfate precipitation, ion exchange, and gel permeation chromatographies were 22.7%, 15.3%, and 4.4%, respectively. There were two kinds of protease inhibitors on the gel permeation chromatography pattern in which their molecular weights were estimated to be 66,700 and 16,000 Da. respectively. Both were classified as a cysteine protease inhibitor because of inhibiting papain, one of cysteine proteases.

Section 2. Purification, Characterization and Inhibitory Activity of Glassfish (*Liparis tanakai*) Egg Low Molecular Weight Protease Inhibitor

Two protease inhibitors with 67 and 18 kDa were purified from the egg of glassfish(*Liparis tanakai)* by affinity chromatography. The yield and purity of 18 kDa protease inhibitor were 0.25% and 49.69 folds, respectively. The amino acid sequence of 18 kDa inhibitor was N-his-ala-asn-arg-val-met-pro-glu-met-asn-met-glu-tyr-met-glu-ala-C. The glassfish protease inhibitor was non-competitive inhibitor against papain with Ki of 4.44 nM. This inhibitor was stable at 50-65 ℃ and pH 8. Glassfish egg protease inhibitor inhibited cathepsin more effectively than egg white protease inhibitor, while reversed on papain. Glassfish egg protease inhibitor is therefore classified into a cysteine protease inhibitor.

Section 3. Purification, Characterization and Inhibitory Activity of Glassfish

(*Liparis tanakai)* Egg High Molecular Weight Protease Inhibitor

Two protease inhibitors of 67 and 18 kDa, respectively, were purified from glassfish(*Liparis tanakai*0 eggs by affinity chromatography and electro-elution method. The higher molecular weight (HMW) protein was purified with a specific inhibitory activity, yield and purity of 18.46 U/mg, 0.07%, and 131.86 fold, respectively, and was further characterized: Optimal temperature and pH for inhibitory activity of HMW glassfish egg protease inhibitor were 40 ℃ and pH 6, respectively, it was stable between 5 ℃ and 50 ℃ in the pH range of 5-6 with maximal stability at pH 6. It was shown to be a competitive inhibitor against papain with an inhibition constant (Ki) of 97.02 nM. Moreover, the 67 kDa protein inhibited cathepsin, a cysteine protease, more effectively than did egg white protease inhibitor. HMW glassfish egg protease inhibitor is classified as a member of the family III (kininogen).

Section 4. Characteristics of the Protease Inhibitor Purified from Chum Salmon

Protease inhibitor of 72.6 kDa was successively purified from chum salmon (*Oncorhynchus keta*) eggs by ion exchange, gel permeation, and affinity chromatographies. Protease inhibitor was purified with yield and purification fold of 1.50% and 58.11, respectively. SDS-PAGE results showed purified protease inhibitor consisted of two protein subunits of 54.0 and 18.6 kDa. Chum salmon inhibitor exhibited stability between 20 and 40 ℃ in weak acid environment (pH 6), and inhibited papain and cathepsin, members of cysteine protease, but not chymotrypsin. The protein inhibited cathepsin more effectivelythan did egg white protease inhibitor, whereas the reverse was true for papain. These results indicate chum salmon egg inhibitor is heterodimer, thus the inhibitor was classified as cysteine protease inhibitor.

Section 5. Characteristics of the Protease Inhibitor Purified from Fish Eggs

The protease inhibitor was purified from five different fish eggs. The molecular weights of Pacific herring, chum salmon, pond smelt, glassfish, and Alaska pollock egg protease inhibitors were 120, 89, 84.5, 17, and 16.8 kDa, respectively. The specific inhibitory activity of glassfish egg protease inhibitor was the highest followed by Pacific herring and Alaska pollock in order. The specific inhibitory activity and purity of glassfish egg protease inhibitor were

19.70 U/mg and 164.70 folds, respectively. Glassfish egg protease inhibitor was reasonably stable at 50-65 oC and pH 8. Inhibitor constant (*Ki*) of glassfish egg protease inhibitor was 4.44 nM.

Section 6. Purification of glassfish egg protease inhibitor from recombinant *E. coli.*

There is a strong demand to prevent the deterioration of surimi based product or fish meat by inhibiting digestive fish muscle proteases such as cathepsin and alkaline proteases, etc. The best way to inhibit the fish muscle proteases is to use the natural inhibitor. However, purification of inhibitor from natural resources is very low in recovery, which consequently limits its application potential. Large-scale production of protease inhibitor for food industrial use and biotechnique is highly demanded. In this study the glassfish egg protease inhibitor was successfully cloned in *E. coli*. and the recombinant protease inhibitor was purified and its activity was compared.

Specific inhibitory activity of recombinant protease inhibitor, 7.117 U/mg, was lower than 19.70 U/mg specific inhibitory activity of natural protease inhibitor from glassfish egg. Because 7 deduced amino acids sequence of recombinant protease inhibitor was changed, especially Met and Asn residues, it might cause the decrease in inhibitory activity.

Section 7. Characterization of the salmon (*Oncorhynchus keta*) egg protease inhibitor in the recombinant yeast

Recombinant chum salmon cystatin (RC) was overexpressed by *Saccharomyces cerevisiae* YPH 499 incorporating pYES2/NT_C. SC medium minus uracil was used for selection and cultivation of the recombinant yeast. The optimal conditions for the production of RC from recombinant yeast were pH of 5.5 and induction time of 12 h, respectively. After cultivation and lysis of the recombinant yeast, RC was purified by His-select nickel affinity chromatography with a yield of 61 % and purity of 5.60 fold. The molecular weight of RC was around 35 kDa based on SDSPAGE. RC showed high inhibitory activities against papain and cathepsin L, and stabilities against heating and weak acidic pH.

Section 8. Application of recombinant chum salmon cystatin to Alaska pollock

(*Theragra chalcogramma*) surimi to prevent gel weakening

Recombinant chum salmon cystatin (RC) expressed in *Saccharomyces cerevisiae* was purified by His-select nickel affinity chromatography. The specific inhibitory activities of RC against papain and cathepsin L were 7.45 and 10.24 U/mg, respectively. RC was stable over pH 5.0 ~ 7.0 and at temperature below 65℃. RC was used to prevent the gel weakening of Alaska pollock surimi. RC at 100μg/g showed the highest inhibitory activity against the autolysis of surimi based on the analysis of TCA-soluble peptides. As the concentration of RC increased, both the breaking force and deformation of modori gel greatly increased ($P < 0.05$). The addition of RC resulted in less expressible drip, which was coincided with the increase of whiteness. More myosin heavy chain (MHC) was retained as the addition of RC increased. Therefore, RC could prevent the degradation of proteins in Alaska pollock surimi was better than egg white (EW). Thus, RC could be applied to Alaska pollock surimi to prevent the gel weakening and RC at 100 μg/g was the optimal concentration.

Section 9. The inhibition of recombinant cystatin against elastase, collagenase and tyrosinase

Elastase inhibitory activity : No obvious inhibitory activity against elastase was checked at two concentrations of recombinant cystatin.

Collagenase inhobotory activity : At 0.05 mg/mL, the recombinant cystatin showed some inhibitory activity against collagenase from Clostridium histolyticum. As the concentration increased to 0.2 mg/mL, no inhibitory activity could be detected.

Tyrosinase inhibitory activity : No obvious inhibitory activity against mushroom tyrosinase was determined at two different concentrations of recombinant cystatin.

Section 10. Characterization of rainbow trout plasma

Effect of fish plasma on textural properties of surimi gel : Surimi gel at 1 mg/g of fish plasma showed the highest breaking force which was around twice of the blank. At addition of 0.75 mg/g, the highest deformation was obtained. The breaking force and deformation were both higher than those of the addition

of egg white with 2 mg/g.

Effect of fish plasma on whiteness and expressible moisture of surimi gels : Texture and color were main effectors determining the market value of surimi-based products. With the addition of fish plasma, the whiteness of surimi gel increased, which was consistent with the decrease of expressible moisture (Table 2). Fish plasma at 0.75 mg/g showed the highest whitening effect, Further addition would decrease the whiteness. The effect of fish plasma was better than that from 2 mg/g of egg white powder addition.

Effect of fish plasma on protein degradation in surimi gels : Below 0.75 mg/g, the fish plasma could inhibit the degradation of myosin heavy chain, further addition will increase the degradation of MHC.

Chapter 2. Mass production and optimization of manufacturing process of protease inhibitor

Section 1. Characterization of protease inhibitor purified from laver and sea lettuce

1. Laver protease inhibitor

Laver protease was extracted with ethanol or sonication and further purified by ion exchange chromatography. The yield of protease inhibitor extracted with ethanol was different depended on the concentration of ammonium sulfate. The concentration of protease inhibitor, 45 mg, was higher in 0-20% ammonium sulfate, but its inhibitory activity, 0.04 unit, was very low. The inhibitory activity against papain, 16 units/mg, was highest in 40-60% ammonium sulfate. Laver protease extracted by sonication with water had similar concentration in the all range of ammonium sulfate. The inhibitory activity against papain was not in 60-80 and 80-100% ammonium sulfate and was similar to ethanol extract in other range of ammonium sulfate. The highest inhibitory activity of laver protease inhibitor against papain was 0.09 unit/mg.

2. Sea lettuce protease inhibitor

Large amount of sea lettuce protease inhibitor, 12 mg/mL, was extracted with ethanol in 0-20% and 20-40% ammonium sulfate, but less than 5 mg/mL in other ranges of ammonium sulfate. The inhibitory activity of sea lettuce

protease inhibitor against papain was very low regardless of its concentration. Its inhibitory activity was 21 units/mg in 80-100% ammonium sulfate. Hence protease inhibitor in 80-100% ammonium sulfate further purified. Two peaks were purified by ion exchange chromatography, in which small peak showed higher inhibitory activity against papain.

Section 2. The characteristics and inhibitory activity of seaweed protease inhibitor

The molecular weight of laver protease purified with ethanol and sonication, and further by ion exchange chromatography was 66 kDa, which is much higher than 13 kDa of cystatin. Based on high molecular weight and high concentration of (+) charge, the molecular weight of laver protease inhibitor might be much higher than that of egg white cystatin.

Seaweed protease inhibitor showed different inhibitory activity depended on ammonium sulfate concentration, temperature, and pH. The inhibitory activity of protease inhibitor extracted in 40-60% ammonium sulfate was relatively high over all experimental temperatures and highest at pH 6.0. Seaweed protease inhibitor was stable over 25-37 ℃ and pH 4.0-6.0. The inhibitory activity of seaweed protease inhibitor was higher at lower temperature compared to that of agricultural and livestock protease inhibitors.

Section 3. Optimization of the culture condition and scaling-up fermentation for recombinant chum salmon cystatin production by *S. cerevisiae*

The culture condition for growth and induction of *S. cerevisiae* YPH 499 to produce recombinant chum salmon cystatin (RC) was optimized on the basis of former experiments in shake flask. Response surface methodology (RSM) method was applied to carry out the optimization for the recombinant yeast cultured in shake flask. Three significant independent variables with five levels, pH of medium, inducing time, and inducing assistant (YNB + amino acids + adenine) amount were studied in this work. Experimental results were approximated to a second-order model with the principal effects of pH of the three factors considered being statistically significant ($P < 0.05$). pH 5.70, inducing time of 6.68 h, and inducing assistant of 5.6 g/L with galactose maintained at 2% were the desirable conditions for enhanced RC production from the recombinant yeast

based on a desirability function. Thereafter, the effect of agitation speeds and aeration rates were studied for the recombinant yeast cultured in a 14-L fermentor upon the optimized conditions above. The highest yield of RC to 0.56 U/mL was obtained with agitation at 350 rpm and aeration at 1.0 vvm. Addition of cold alcohol to 45% (v/v) could help the purification of RC from the recombinant yeast.

CONTENTS

목 차

제 1 장 서 론

제 1 절 연구목적

최근 국민소득의 증대에 따라 국내에서는 고소득사회에 따른 건강요구형 식품의 수요가 날로 급증할 것으로 예상된다. 또한 축육을 주식으로 하는 서양식 식생활은 여러 가지 성인병 및 암을 유발할 뿐 아니라 최근에는 광우병 등의 영향을 소비자들이 기피하고 있는 실정이다. 따라서 상대적으로 수산식품의 소비가 급격하게 증가함에 따라 유통기간 연장 등 수산식품에 관한 관심이 증가하고 있다. 수산식품 중 맛살류(어묵), 젓갈류의 경우 일정한 유통기간을 가질 수밖에 없는데, 이것은 수산물의 근육이나 조직 자체에 함유되어 있는 내인성 효소 및 미생물이 분비하는 외인성 효소의 작용이 매우 중요한 작용을 하는 것으로 연구되었다 특히 맛살류나 젓갈류의 주원료는 어류이므로 단백분해효소의 작용이 매우 중요하다. 단백분해효소는 맛살류나 젓갈류에 작용하여 고유의 풍미나 독특한 조직감이 사라지는 등 품질열화가 일어나게 한다. 따라서 이들 제품의 유통기간 연장 및 품질 향상을 위해서는 효과적으로 단백분해효소의 활성을 억제하는 것이 필수적이다. 그러나 현재 농·축산물 유래 천연 단백분해효소 저해제에 대한 연구에 비하여 수산물 유래 저해제에 대한 연구는 초보단계에 머물러져 있으며, 수산물 유래 단백분해효소 저해제의 대량생산에 대한 연구는 아직 시작단계에 머물러져 있는 실정이다. 또한 생물공학기술을 통한 수산물 유래 단백분해효소 저해제의 생산기술은 아직 개발단계에 있으며, 대부분의 저해제의 경우 아직 유전자의 동정조차 이루어지지 않고 있는 실정이다.

또한 천연효소 저해제가 첨가된 포장 맛살 및 젓갈제품의 품질변화 분석 및 저장기간 예측에 대한 연구는 거의 없다. 즉 저장조건이 포장 맛살 등 수산연제품 조직의 단백분해효소 활성과 효소-저해제간의 반응활성에 미치는 영향은 거의 보고된 바가 없으며 젓갈의 저장과 유통에 대한 연구는 아직 체계적인 연구가 없는 실정이다. 따라서 어란(연어, 청어, 명태, 까나리의 알 등), 해조류(녹조, 홍조, 갈조 등) 등 수산 동·식물로부터 유전자 조작을 통하여 천연단백분해효소 저해제를 대량생산하는 기술을 개발하고, 맛살류, 젓갈류 등 수산식품의 품질의 고급화 및 유통기간을 연장을 도모함으로써 소비확대 및 수출 증대 등 수산업 발전에 기여하는 것이 본 연구의 목적이라 할 수 있겠다.

제 2 절 연구개발 필요성

1. 연구개발의 필요성

가. 기술적 측면

O 단백분해효소에 의한 품질열화는 수산식품의 유통기간 결정에 매우 중요하다.
O 수산식품에 존재하는 단백분해효소의 활성억제를 위해서는 수산물로부터 천연 단백분해효소 저해제를 개발하여 이용하는 것이 가장 효과적이다.
O 천연 저해제의 상품화를 위해서는 저해제의 대량생산기술 개발 및 저해제가 첨가된 수산식품의 품질분석과 저장기간의 예측이 필수적이다.

수산식품 중 조미가공품 생산량은 1998년 6,466, 1999년 8,072, 2000년 14,532 M/T로 생산량이 급격하게 늘고 있으며, 정확한 통계량은 나와 있지 않으나 맛살류(어묵) 생산량이 대부분을 점하고 있는 것으로 알려져 있다. 수산발효식품인 젓갈류는 1998년 42,834, 1999년 60,6070, 2000년 56,974 M/T으로 원료공급에 따라 약간의 변동이 있으나 생산량이 증가하고 있는 추세에 있다. 그러나 맛살류와 젓갈류를 비롯한 모든 동물성 혹은 동물성 발효식품은 일정한 유통기간을 가질 수밖에 없는데, 여러 가지 요인들이 있으나 이들 식품의 유통기간 결정에는 동물의 근육이나 조직 자체에 함유되어 있는 내인성(endogenous) 효소 및 미생물이 분비하는 외인성(exogenous) 효소의 작용이 매우 중요하다. 특히 수산식품인 맛살류 및 젓갈류의 주원료는 어류이므로 단백분해효소의 작용이 매우 중요하다. 맛살류는 가열공정 동안 내온성 단백분해효소에 의해 단백질이 분해되어 독특한 조직감을 구성하는 gel 구조가 파괴되며, 젓갈류는 자가소화효소 및 미생물이 분비하는 단백분해효소에 의해 어느 정도 기간이 지나면 과숙성되어 고유의 풍미가 사라지는 등 품질열화가 일어난다. 그러므로 이들 제품의 유통기간 연장 및 품질향상을 위해서는 효과적으로 단백분해효소의 활성을 억제하는 것이 필수적이다.

단백분해효소의 활성을 억제하기 위하여 여러 방법들이 적용되어 왔으나 천연 저해제를 이용하는 것이 가장 바람직한 방법의 하나로 알려지고 있다. 그러나 현재 맛살류의 gel 파괴를 방지하기 위하여 연구되고 있는 천연 단백분해효소 저해제의 대부분은 대두를 비롯한 곡류 및 소, 돼지의 혈장 등의 농·축산물에서 추출되며 수산식품에 존재하는 단백분해효소에 완벽하게 작용하지 않는다. 따라서 맛살류, 젓갈류와 같이 원료가 수산물인 식품의 유통기간 연장과 품질향상을 위해서는 수산물 유래 단백분해효소들의 활성억제에 효과적인 천연 저해제를 개발하는 것이 시급하며, 이를 위해서는 수산물에 천연적으로 존재하는 단백분해효소 저해제를 개발, 산업화하는 것이 가장 바람직하다. 그러므로 본 연구에서는 수산식품의 단백분해효소에 선택적으로 작용하는 저해제를 수산동물 (어란, 강장동물) 및 해조류에서 추출한 후 이를 유전자 조작과 재조합 미생물의 개발을 통하여 대량 생산할 수 있는 기술을 확립하고자 한다.

나. 경제・산업적 측면

WTO 체제 출범 후 각종 농・수・축산물의 수입이 지속적으로 증가하고 있으며, 식품관련분야 기술의 발달로 고가의 식품소재나 건강보조식품의 수입도 꾸준히 증가하고 있다. 이러한 다양한 식품 수입개방에 대응하기 위한 효과적인 방안의 하나는 우리전통식품의 우수함을 밝히고 이들의 품질과 안정성을 강화하여 외국식품의 수입억제 및 국내 식품산업의 해외 진출에 기여하는 것이다. 조미연제품인 맛살류(surimi-based product) 및 전통수산발효식품인 젓갈류는 우리나라 수산식품의 주 품목으로 그 상품성을 인정받으며 꾸준한 생산량의 증가를 보이고 있다. 이들 주요 수산식품의 품질향상 및 유통기간 연장을 위하여 수산식품 품질열화의 주원인 중의 하나인 내인성 및 외인성 단백분해효소의 활성을 저해할 수 있는 천연저해제의 개발과 산업화는 신규시장 형성과 함께 관련 산업의 발전을 가져올 뿐만 아니라 우리문화의 우수성도 함께 밝혀내는 매개체 역할을 할 것이다.

다. 사회・문화적 측면

국민소득의 증대에 따라 육류의 소비가 증가하는 등 생활패턴이 달라지면서 고혈압, 동맥경화 등 순환기 계통의 질병 및 암의 발생률이 증가하고 있다. 2001년도 보건복지부의 보고에 의하면 우리국민의 사망원인별 1위는 순환기계 질병 및 암이 비슷하였다고 한다. 따라서, 국민보건의 차원에서 이에 대한 대책이 시급히 요망되며, 의료분야의 대응책 이외에 식생활의 개선을 통한 접근이 필요하다고 본다. 현재 국내에서는 고소득사회에 따른 건강요구형 식품의 수요가 날로 급증할 것으로 예상되며, 이에 대한 식품산업의 대비가 필요한 실정이다. 축육 (소 및 돼지)을 주식으로 하는 서양식 식단은 동맥 경화 등 여러 가지 성인병 및 암을 유발할 뿐 아니라 최근에는 광우병 등의 영향으로 소비자들이 기피하고 있는 실정이며 상대적으로 수산식품의 소비가 급격하게 증가하고 있다. 또한, 갈수록 사라져 가는 우리전통 식문화를 보존하고 전통식품 (수산발효식품)의 우수함을 밝혀내는 것은 국민 건강 증진 뿐만 아니라 문화적인 자부심에도 보탬이 될 것으로 본다.

2. 국내・외 관련연구의 현황과 문제점

O 농・축산물 유래 천연 단백분해효소 저해제에 대한 연구이외에, 수산물 유래 저해제에 대한 연구는 아직 초보단계에 머물러 있다.
O 유전자 조작을 이용한 수산물 유래 단백분해효소 저해제의 대량생산에 대한 연구는 아직 시작단계에 머물고 있다.

가. 단백분해효소 저해제

수산물의 주 내인성 분해효소는 cysteine계 단백분해효소로 cathepsin (Seymour et al., 1994) 및 열에 안정한 알칼리성 단백분해효소 (Boye and Lanier, 1988)이다. 맛살류의 단백분해효소에 관한 연구는 주로 알칼리성 효소의 활성을 억제하는 방향으로 연구되어 왔는데, 그 이유는 어육의 사후에 일어나는 근육조직의 분해에 관계되는 많은 단백분해효소(산성, 알칼리성, 중성) 가운데 알칼리성 효소가 맛살류의 gel weakening (modori)의 주원인으로 작용하기 때문이다 (Wasson, 1992; An et al., 1994). 즉, 맛살류는 탄력성(gelation)을 얻기 위하여 가열처리를 하는데, 가열공정 동안 알칼리성 cysteine계인 내열성 단백분해효소에 의한 gel 파괴가 발생하므로, 저해제(inhibitor)를 첨가하여 효소활성을 억제하여 gel강도를 최대화하기 위한 연구가 활발하게 이루어져 왔다.

일반적으로 천연 단백분해효소 저해제는 주로 대두 (Hines et al., 1991; Dipietro and Liener, 1989; Jo et al., 1989), 달걀흰자 (Barrett, 1981; Nicklin and Barrett, 1984), 쌀 (Kondo et al., 1990; Abe et al., 1987), 감자 (Pompe-Novak et al., 2002), 수수 (Joshi et al., 1998), 우유(초유) (Kirihara et al., 1995), 소와 돼지의 혈장 (Morrissey et al., 1993; Weerasinghe et al., 1996; Benjakul and Visessanguan, 2000) 등의 농·축산물에서 생산되며, 세균 (Kim and Lee, 1990; Kim et al., 1991), 원생동물 (Hellberg et al., 2002) 등에서도 분리·정제되어 이용되고 있다 (표 1). 그러나 이들 저해제는 단백분해효소를 저해하여 gel 강도를 강화시키는 이점이 있지만, 원하지 않는 색상이나 맛을 내는 부작용이 있으며, 또한 이들 저해제의 효과는 어종이나 맛살의 제조방법에 따라 많은 차이가 난다 (Akazawa et al., 1993; Reppond and Babbitt, 1993).

표 1. 천연 단백분해효소 저해제 추출원료

미생물		*Streptomyces* sp, *Entamoeba histolytica*
동물		소, 돼지의 혈장, 우유, 달걀 흰자
식물		대두, 쌀, 감자, 수수
수산물	어류	말미잘, 꽃게, 송어알, 연어알, 잉어, zebrafish
	해조류	*Padina* sp., *Acantophora* sp., *Cladosiphon* sp.

나. 수산물 유래 단백분해효소 저해제

일반 단백분해효소 저해제의 단점을 극복하기 위해서는 수산 식품의 단백분해효소에 선택적, 효과적으로 작용하는 저해제를 농·축산물이 아닌 수산물에서 생산하는 것이 가장 바람직하다. 또한, 대표적인 전통 수산발효식품인 젓갈류의 경우, 어느 정도 숙성이 지난 다음 자가분해효소 및 미생물이 분비하는 효소에 의하여 품질 열화가 일어나는데, 이러한 효소의 작용에 대한 연구 및 이들 저해제를 이용한

품질열화의 방지와 저장성 증대에 대한 연구는 거의 없는 실정이다. 수산물에서의 단백분해효소 저해제 분리·정제에 대한 내용은 몇몇 보고에 국한되어 있다.

수산동물에서 기인한 단백분해효소 저해제의 경우, 말미잘 (Rogelj et al., 2000), 꽃게 체액 (김 등, 1993a; 김 등 1993b), 송어알 (Li et al., 1998, 2000), 연어알 (Yamashita and Konagaya, 1991, 1996), 잉어 (Tsai et al., 1996), Zebrafish (Gong et al., 1997) 등에서 성공적으로 분리·정제하였다는 연구가 보고되어 있으며 (표 1), 이들 저해제를 합성하여 생산하려는 연구 (Walker et al., 2000)도 보고되고 있으나 아직 초기단계에 머물러있는 실정이다. 특히, 젓갈류의 주 생산품인 명란 및 창란의 원료는 명태인데, 아직까지 명란에서 단백분해저해제를 연구한 보고는 없는 실정이다.

해조류에서 기인한 단백분해효소 저해제에 대한 연구는 극히 드문 실정이다. 해조류의 일종인 Padina gymnospora와 Acantophora spicifera에서 미량의 trypsin inhibitor들이 발견되었으며 (Perez-Lorenzo et al., 1998), 갈조류 등에서 추출된 dextran sulfate, carrageenan, Fucus fucoidan 등의 sulfated polysaccharides (황산다당류)가 pepsin (Shibata et al., 2000), protein kinase (Religa et al., 2000), heparase (Hershkoviz et al., 1995), HIV (Schaeffer and Krylov, 2000), 및 endo-(1→3)-β-D-glucanase (Yermakova et al., 2002)의 활성을 억제함이 보고되었다. 일반적으로 녹조류와 홍조류에는 상당량의 단백질이 (10-47%, dry basis) 함유되어있는데, 녹조류로서 파래김의 원료가 되는 Ulva pertusa의 경우 단백질 함량이 20-26%, 홍조류에 속하며 일반 김의 원료가 되는 Porphyra tenera의 단백질 함량은 약 47% 정도로 콩류보다도 높다 (Fleurence, 1999). 또다른 홍조류인 Palmaria palmata에도 약 35% 정도로 높은 함량의 단백질이 존재한다 (Fleurence, 1999). 녹조류나 홍조류의 높은 단백질 함량을 고려해 볼 때 이들 해조류에는 상당량의 단백분해효소 저해제가 존재함이 예상되나 아직 이에 대한 연구는 이루어지지 않고 있다.

다. 유전자조작에 의한 단백분해효소 저해제의 생산

유전자 조작을 이용하여 단백분해효소 저해제를 대량생산하려는 연구는 주로 ***육상동물의 저해제,*** 즉 human cystatin C (Abrahamson et al., 1987), human cystatin S (Isemura et al., 1986), chicken cystatin C (Colella et al., 1989), mouse cystatin C (solem et al., 1990) 및 rat cystatin (Cole et al., 1989) 등에 국한되어 연구되어 왔으며, 이를 파리에 의해 전파되는 Leishmania병 (Selzer et al., 1997;

Sakanari et al., 1997) 및 모기에 의해 전파되는 Trypanosoma병(Scory et al., 1999; Troeberg et al., 1999) 등의 치료제로서 개발하려는 연구도 진행되고 있다. 최근 들어 무지개송어 (Li et al., 2000), 참연어 (yamashita and Konagaya, 1996), 잉어 (Tsai et al., 1996) 및 zebrafish (Gong et al., 1997) 등 수산물의 cystatin 저해제를 유전자 조작과 재조합 미생물을 이용하여 생산하려는 연구들이 보고되었다. 한 예로서, 말미잘의 cysteine계 단백분해효소 저해제인 equistatin의 유전자를 함유한 재조합 E. coli와 Pichia pastoris는 각각 1mg/L와 25mg/L의 수율로 저해제를 생산하였다 (Rogelj et al., 2000). 그러나 이러한 생물공학기술을 통한 저해제의 생산기술은 아직 개발단계에 있으며, 일부를 제외한 대부분의 수산물 유래 단백분해효소 저해제의 경우 아직 유전자의 동정조차 이루어지지 않고 있다. 따라서, 효과적인 천연 단백분해효소 저해제를 우리 수산물에서 동정하고 이를 유전자 조작 및 재조합 미생물을 이용하여 대량생산하는 기술을 확립하는 것은 우리 수산기술 및 산업의 경쟁력 강화와 활성화에 매우 중요하다.

3. 앞으로 전망

환경 및 사회변화와 함께 고혈압, 신장병 등 각종 성인병과 암 등의 발생율 증가와 노령화 추세로 인하여 건강 욕구를 충족시킬 수 있는 식품의 수요가 급격히 증가하고 있는데, ***성인병 및 암을 예방할 수 있는 기능성 물질(ω-3 지방산, 타우린, 키토산 등)이 수산식품에 많이 함유***되어 있다는 사실이 알려진 이래로 수산식품의 소비가 계속 증가하고 있다. 단백분해효소 저해제는 맛살과 젓갈의 품질열화를 막을 수 있는 가장 이상적인 첨가제의 하나이며, 품질열화의 mechanism이 밝혀질수록 저해제의 사용은 급격하게 증가할 것으로 보여진다. 수산식품에는 그동안 한정적으로 농·축산물 유래 저해제를 생산하여 맛살류 생산에만 사용되어 왔으나, 수산식품에는 그 효능이 약하여 ***수산식품에 선택적으로 작용할 수 있는 저해제의 개발이 요구***되어 왔다. 만약 수산식품에 선택적으로 작용할 수 있는 저해제가 개발되어진다면 그 용도는 대폭 확대될 것으로 예상된다. ***맛살류***는 이미 산업화되어 대량생산되고 있으므로 유전자 조작에 의한 천연 단백분해효소 저해제와 이를 이용한 효과적인 저장기술의 개발은 품질과 유통기간을 향상시켜 수출증가 등으로 이어지며 막대한 이익을 가져올 수 있다. 전통발효식품인 ***젓갈류***는 우리 고유의 독특한 풍미와 우수한 영양성에도 불구하고 아직 생산시설이 영세한 경우가 많고, 품질보존을 위하여 고염의 식염을 사용하는 관계로 일부 소비자들이 기피하고 있다. 따라서 저염 젓갈에 대한 연구가 활발한데, 저염으로 인한 저장성 감소를 극복할 수 있는 방법에 대한 연구가 함께 이루어져야 경쟁력이 있는 수산식품으로 산업화될 수

있을 것이다. 천연 단백분해효소 저해제와 이를 이용한 ***수산식품 (젓갈 및 맛살류) 저장기술의 개발은 수산식품의 품질열화 및 저장성 문제를 효과적으로 해결***하여 수산식품 시장의 안정화와 증대뿐만 아니라 관련산업의 발전에도 크게 이바지 할 수 있을 것이다.

4. 기술도입의 타당성

현재 국내의 아미노산 구조분석기술, 효소와 저해제의 활성 평가기술, 및 단백질 분리·정제기술 등은 외국의 경우에 비하여 손색이 없다. 또한 단백질의 유전자 복제 기술 및 이를 이용한 재조합 미생물의 개발과 발효기술도 이미 경쟁력이 있는 수준에 올라있다. 이러한 기술을 이용하여 수산식품에 적합한 새로운 단백분해 저해제를 개발·생산하거나 새로운 의약제품의 생산을 유도하여 우리나라의 해양 및 관련 산업의 확장에 기여하는 것은 기술경쟁사회에서 매우 중요한 의미를 지닌다. 국내의 식품저장, 품질분석에 대한 기술도 비록 생물, 유전자공학 분야에 비하여 상대적으로 그 발전이 더딘 실정이긴 하나, 꾸준히 연구, 개발되어 상당한 수준에 와 있다. 특히 젓갈과 같은 우리 고유의 수산식품들에 대해서는 저장, 품질분석 등에 대한 자체연구를 통하여 식품개방에 대한 문화적·경제적 경쟁력을 확립해야한다고 본다. 그러므로 외***국으로부터의 기술도입은 현 시점에서는 필요하지 않을 것***으로 사료된다.

제 3 절 연구개발 범위

1. 천연 단백분해효소 저해제의 대량생산 및 수산식품에의 응용 기술 개발

가. 수산동물 (명태, 도치, 도루묵, 물곰치, 빙어, 연어) 알의 단백분해효소저해제의 검색

수산동물의 알을 이용하여 단백분해효소를 column chromatography로 분석하여 분리•정제하여 단백분해효소 저해활성 측정 방법 확립 (Papain activity 저해 활성 측정)하고 HPLC, SDS-electrophoresis을 행하여 단백분해효소 저해제의 순도 및 분자량 측정 을 확립하였다.

나. 수산동물에 함유된 천연 단백분해효소저해제의 분리 및 정제

단백분해효소저해제 정제 및 특성실험 완료하였으며, 저해효과 및 저해기작 분석 완료하였다. 합성 및 천연 물곰치알 단백분해효소저해제의 효과 및 저해기작 실

험을 완료하였으며, Cathepsin 및 trypsin에 대한 물곰치알 저해제의 저해활성을 비교분석하였다. 상업용 제품(Egg white 및 potato inhibitor)과의 비교 실험완료

다. 분리된 천연 효소저해제의 효과 및 저해기작 평가

Ki and Vmax value 측정에 의한 저해기작을 평가하였고, 온도, pH 및 수분활성의 영향 분석을 완료하였다.

라. 효과가 우수한 수산물 유래 저해제의 아미노산배열 분석, 유전자 동정 및 PCR 을 통한 유전자 복제

물곰치 저분자 단백분해효소저해제의 아미노산배열 분석, 유전자 동정 및 PCR을 통한 유전자 복제를 완료하였으며, E. coli 균에 대한 cloning을 완료하였다. Cloned E. coli 균으로부터 재조합 단백분해효소저해제의 최적 배양조건을 확립하여 재조합 단백분해효소저해제의 정제 및 활성 비교를 완료하고, Calpain inhibitor의 저해활성 비교실험을 완료하였다.

마. 연어알 단백분해효소 저해제의 재조합 효모의 발효공정 및 효소저해제 생산의 최적화

효모에 발현된 연어알 단백분해효소 저해제의 배지 조성 및 배양조건의 최적화를 확립하였다.

바. 재조합 미생물의 발효를 통한 천연 효소저해제의 대량생산공정과 분 리 정제기술의 확립

연어알 단배분해효소 저해제의 정제 방법 및 저해활성을 연구하였다.

사. 재조합 효소저해제가 함유된 수산식품의 품질향상

단백분해효소저해제를 첨가한 surimi를 제조하여 품질 특성 실험을 하였으며, 화장품 관련 효소에 대한 저해활성 실험을 완료하였다.

2. 단백분해효소 저해제의 대량 생산 및 생산 공정의 최적화

가. 해조류(홍조 및 녹조)로부터 단백분해효소 저해제를 검색

해조류 2종(홍조인 방사무늬김, 녹조인 창자파래)으로부터 단백질분해효소 저해제를 확인하고 단백질분해효소 저해활성을 측정하였다.

나. 해조류에 함유된 단백분해효소저해제의 분리 및 정제

홍조류(P. yezoensis and tenera) 및 녹조류(E. intestinalis)의 단백분해효소저해

제 효과의 비교 평가를 행하였으며, *P. yezoensis*로부터의 단백질분해 효소저해제 분리공정효율을 평가하였다.

다. 해조류 효소저해제의 효과 및 저해기작 평가

*P. yezoensis*의 단백분해효소저해제의 특성을 온도 및 산도(pH)에 따라 beef plasma protein, egg white, potato powder 등 기존의 농·축산물 유래 저해제와 비교 평가하였다.

라. 물곰치 단백분해효소 저해제의 N-terminal 분석 및 합성

물곰치 알로부터 유래되는 단백분해효소저해제의 N-terminal을 근거로 peptide 합성을 행하였다.

마. 연어알 단백분해효소저해제의 재조합 벡터 및 재조합 효모 개발

Cystatin 발현 벡터 pYES2/NT_C (cystatin)를 효모(S. cerevisiae YPH499)에 형질전환하였으며, 재조합 벡터를 제작해 효모에서 발현하였다.

바. 물곰치알 재조합 벡터 및 재조합 효모 개발

-재조합 벡터를 제작해 물곰치 유래 단백분해효소저해제의 형질이 전환된 효모 개발하고 재조합 단백분해효소 저해제의 정제 및 활성을 평가하였으며, 형질 전환된 효모의 대량생산방법을 연구하였다.

제 2 장 국내외 기술개발 현황

제 1 절 국내 기술현황

1. 단백분해효소 저해제

곡류, 육상동물, 수산동물, 해조류로부터 새로운 단백분해효소 저해제를 추출, 개발한 연구는 국내에서는 아직 보고된 바 없다. 미생물 유래 단백분해효소 저해제에 대한 연구로는, Kim and Lee (1990) 및 Kim et al. (1991)이 *Streptomyces spp.*에

서 세포외 (extracellular) thiol계 단백분해효소 저해제를 분리·정제하여 이의 생리학적 역할을 규명한 연구가 있으나, 활발하게 이루어지지는 않고 있다.

2. 단백분해효소 저해제의 생물공학적 대량 생산

유전자 조작 (gene cloning, genetic engineering, recombinant DNA technology), 발효 (fermentation), 공정 최적화 (optimization), 분리 및 정제 (separation and purification) 등의 생물공학 기술을 이용한 제품생산은 국내에서도 활발하게 이루어지고 있으며 이들 기술은 일반화한 상태이다. 그러나 단백분해효소 저해제의 생물공학적 생산에 대한 연구는 아직 보고되어 있지 않다.

3. 수산식품의 단백분해효소

가. 맛살류의 단백분해효소

맛살류 수산제품은 국내에서 큰 시장을 형성하고 있으나 이에 대한 국내 전문가는 극소수이며 맛살류의 품질열화를 방지하기 위한 연구의 기초가 되는 단백분해효소에 대한 연구는 아직 초보단계에 머물러있다.

나. 젓갈류의 단백분해효소

젓갈류 유래 단백분해효소에 대한 연구는 이 (1968), 차 및 이 (1989), 차 등 (1988), 김 등 (1994)의 연구 이외에는 찾아보기 힘들다. 이들 연구는 완전 정제된 효소에 대한 것이 아니라 부분 정제된 조효소에 대한 기초 연구에 불과하다. 또한, 단백분해효소의 origin 및 이들 효소를 저해할 수 있는 방법에 대한 연구는 아직 없는 실정이다.

4. 단백분해효소 저해제에 의한 수산식품의 품질개선 및 유통기간 연장에 대한 연구

가. 저해제를 수산식품 제조시 첨가하는 연구

(1) 맛살류에 대한 저해제의 이용

단백분해효소 저해제가 맛살의 품질에 미치는 영향에 관한 국내 연구는 아직 보고되지 않았다.

(2) 젓갈류에 대한 저해제의 이용

수산발효식품인 젓갈류는 자가소화효소 및 미생물이 분비하는 단백분해효소에

의해 육질이 분해되어 독특산 감칠맛을 나타나게 하는데 이들 효소의 작용이 너무 강하거나 오래 지속이 되면 품질열화를 가져온다. 특히, 명란젓인 경우 명란의 껍질막 (egg membrane)의 연화가 크면 명란 전체의 품질열화를 가져오기 때문에 이들을 분해하는 단백분해효소의 활성을 억제하기 위한 저해제의 연구는 시급한 실정이나 아직 이에 대한 국내 연구보고는 없는 실정이다.

제 2 절 국외 기술현황

1. 단백분해효소 저해제

가. 곡류 단백분해효소 저해제

곡류 유래 단백분해효소 저해제는 해충이나 곤충의 단백분해효소 활성을 억제하여 곡식의 피해를 줄이려는 방향으로 연구되어왔는데, 대두 (Hines et al., 1991; Dipietro and Liener, 1989; Jo et al., 1989), 감자 (Pompe-Novak et al., 2002), 쌀 (Kondo et al., 1990; Abe et al., 1987), 수수 (Joshi et al., 1998) 등에서 주로 추출된다. 이들 저해제는 대부분 cysteine계 단백분해효소를 저해하는 저해제 (cystatin)로 알려져 있다.

나. 육상동물 단백분해효소 저해제

육상동물 유래 단백분해효소 저해제는 주로 달걀흰자 (Barrett, 1981; Nicklin and Barrett, 1984), 우유 (초유) (Kirihara et al., 1995) 및 소와 돼지의 혈장 (Morrissey et al., 1993; Weerasinghe et al., 1996; Benjakul and Visessanguan, 2000)에서 추출된다. 이들 저해제는 주로 수산연제품인 맛살류 (어묵) (surimi-based product)의 단백분해효소의 활성을 억제하여 품질열화를 억제하려는 목적으로 연구되어 왔다. 그러나 이들 저해제는 단백분해효소를 저해하여 gel 강도를 강화시키는 이점이 있지만, 원하지 않는 색상이나 맛을 내는 부작용이 있으며, 또한 이들 저해제의 효과는 어종이나 맛살의 제조방법에 따라 많은 차이가 난다 (Akazawa et al., 1993; Reppond and Babbitt, 1993).

다. 미생물 단백분해효소 저해제

미생물 유래 단백분해효소 저해제는 세균 (Kim and Lee, 1990; Kim et al., 1991) 및 원생동물 (Hellberg et al., 2002)에서 분리·정제되었다는 보고가 있으며, 주로 세포내의 대사작용에 미치는 영향을 분석하기 위하여 연구되고 있다.

라. 수산동물 단백분해효소 저해제

수산동물 유래 단백분해효소 저해제의 경우, 말미잘 (Rogelj et al., 2000), 꽃게 체액 (김 등, 1993a; 김 등 1993b), 송어알 (Li et al., 1998, 2000), 연어알 (Yamashita and Konagaya, 1991, 1996), 잉어 (Tsai et al., 1996), Zebrafish (Gong et al., 1997) 등에서 성공적으로 분리·정제하였다는 연구가 보고되어 있으며, 주로 단백분해효소 분자의 진화과정을 밝히려는 목적으로 연구되고 있다. 그러나, 아직 젓갈류의 주 생산품인 명란 및 창란 유래 단백분해효소 저해제에 대한 연구는 보고된바 없다.

마. 해조류 단백분해효소 저해제

Perez-Lorenzo et al.(1998)은 해조류의 일종인 Padina gymnospora와 Acantophora spicifera에서 미량의 trypsin inhibitor들을 찾아내었으며, Shibata et al. (2000)은 갈조류에서 추출한 dextran sulfate, carrageenan, Fucus fucoidan 등의 sulfated polysaccharides가 pepsin의 활성을 저해함을 보고하였다. 일반적으로 녹조류와 홍조류에는 상당량의 단백질이 (10-47%, dry basis) 함유되어있는데, 녹조류로서 파래김의 원료가 되는 Ulva pertusa의 경우 단백질 함량이 20-26%, 홍조류에 속하며 일반 김의 원료가 되는 Porphyra tenera의 단백질 함량은 약 47% 정도로 콩류 보다도 높다 (Fleurence, 1999). 또 다른 홍조류인 Palmaria palmata에도 약 35% 정도로 높은 함량의 단백질이 존재한다 (Fleurence, 1999). 녹조류나 홍조류의 높은 단백질 함량을 고려해 볼 때 이들 해조류에는 상당량의 단백분해효소 저해제가 존재함이 예상되나 아직 이에 대한 연구는 이루어지지 않고 있다.

2. 단백분해효소 저해제의 생물공학적 대량 생산

유전자 조작을 이용하여 단백분해효소 저해제를 대량생산하려는 연구는 주로 육상동물의 저해제, 즉 human cystatin C (Abrahamson et al., 1987), human cystatin S (Isemura et al., 1986), chicken cystatin C (Colella et al., 1989), mouse cystatin C (solem et al., 1990) 및 rat cystatin (Cole et al., 1989) 등에 국한되어 연구되어 왔으며 곤충에 의해 전파되는 질병 방지가 주된 목적이다. 최근 무지개송어 (Li et al., 2000), 참연어 (yamashita and Konagaya, 1996), 잉어 (Tsai et al., 1996), zebrafish (Gong et al., 1997), 말미잘 (Rogelj et al., 2000) 등의 수산물에서 유래한 cystatin 저해제를 유전자 조작과 재조합 미생물을 이용하여 생산하려는 연구들이 보고되었다. 그러나 이러한 생물공학기술을 통한 저해제의 생산기술은 아직 개발단계에 있으며, 일부를 제외한 대부분의 수산물 유래 단백분해효소 저해제의 경우 아직 유전자의 동정조차 이루어지지 않고 있다.

3. 수산식품의 단백분해효소

가. 맛살류의 단백분해효소

맛살류의 품질열화를 가져오는 내인성 단백분해효소는 주로 cysteine계 단백분해효소로 cathepsin (Seymour et al., 1994) 및 열에 안정한 알칼리성 단백분해효소(Boye and Lanier, 1988)인 것으로 알려져 있다.

나. 젓갈류의 단백분해효소

젓갈류는 우리 고유의 식품이므로 젓갈류 유래 단백분해효소와 이들이 젓갈류의 품질변화에 미치는 영향에 대한 외국의 연구는 보고된 바 없다.

4. 단백분해효소 저해제에 의한 수산식품의 품질개선 및 유통기간 연장에 대한 연구

가. 저해제를 수산식품제조시 첨가하는 연구

(1) 맛살류에 대한 저해제의 이용

맛살류는 탄력성을 얻기 위하여 가열처리를 하는데, 가열공정동안 알칼리성 cysteine계 내인성 단백분해효소에 의한 자가소화로 인하여 gel의 파괴가 발생한다고 알려져 있다. 따라서 육상생물 유래 단백분해효소 저해제를 첨가하여 제품의 탄력성을 유지하기 위한 연구가 활발하게 이루어져 왔다 (Wasson, 1992; An et al., 1994).

(2) 젓갈류에 대한 저해제의 이용

젓갈류에의 단백분해효소 저해제의 이용에 대한 외국의 연구사례는 보고된 바 없다.

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제 3 장 수산동물 유래 단백분해효소 저해제의 개발 및 수산식품에의 응용

제 1 절 명란 (Alaska pollock egg)으로부터 단백분해효소 저해제 정제 및 특성

1. 서 론

동물성 식품의 유통기간을 결정하는데 있어서 식품의 원료로 사용되어지는 동물의 근육이나 조직자체에 함유되어 있는 내인성 (endogenous) 효소 및 미생물이 분비하는 외인성 (exogenous) 효소의 작용은 매우 중요하다. 특히 수산식품인 맛살류 및 젓갈류의 품질열화의 주원인은 단백분해효소로 알려져 있다. 맛살류의 경우 제품의 원료인 어류의 근육 및 체내에 존재하는 내인성 (endogenous) 단백분해효소가 단백질을 가수분해하여 근육조직의 연화와 파괴를 가져오며 (An et al., 1996), 제품의 겔 (gel)화를 위한 가열공정 동안 내열성 단백분해효소에 의해 단백질이 분해 되어 독특한 조직감을 구성하는 gel 구조가 파괴 (modori) 된다 (Saeki et al., 1995). 또한 젓갈류는 자가소화효소 및 미생물이 분비하는 단백분해효소에 의해 어느 정도 기간이 지나면 과숙성 되어 고유의 풍미가 사라지고 조직감이 저하되는 등의 품질열화가 일어난다 (Cha and Lee, 1994). 그러므로 이들 제품의 유통기간 연장 및 품질향상을 위해서는 효과적으로 단백분해효소의 활성을 억제하는 것이 필수적이다. 단백분해효소의 활성을 억제하기 위하여 여러 방법들이 적용되어 왔으나 천연저해제를 이용하는 것이 가장 바람직한 방법의 하나로 알려지고 있다. 대부분의 단백분해효소는 그에 부합하는 특이적인 저해제(inhibitor)에 의하여 활성이 저해되며, 활성부위의 종류에 따라 cysteine, serine, aspartic acid 및 metallo 단백분해효소로 분류된다 (Kenny, 1999). 이 중 수산물의 주 내인성 분해효소는 cysteine계 단백분해효소로 cathepsin (An et al., 1994) 및 열에 안정한 알칼리성 단백분해효소 (Boye and Lanier, 1988)이다. 천연 단백분해효소저해제는 달걀흰자, 소 혈장, 감자전분 등에서 분리한 것이 있으나 (Weerashinghe et al., 1996), 소 혈장 유래 단백분해효소 저해제는 광우병 문제로 사용이 금지되었으며 아직까지 수산물에서 분리한 것은 많지 않은 실정이다. 미국산 연어란 (Yamashita and Konagaya, 1991) 및 잉어란 (Tsai et al., 1996)에서 단백분해효소 저해제를 분리·정제한 연구가 보고 되었으나 상품화하지는 못하였다. 본 연구에서는 맛살류 및 젓갈의 품질 열화를 방지할 목적

으로 명란 (Egg of Alaska pollock)에서 분리·정제한 단백분해효소 저해제의 특성을 분석하였다.

2. 재료 및 방법

가. 재료

실험에 사용된 명란은 2003년 10월 동해안산 명태 (*Theragra chalcogramma*)에서 채취한 신선한 알로 -40℃에서 동결·저장하여 필요시 해동하여 사용하였다. Papain, azocasein, molecular marker (Sigma MS-70)는 Sigma 사 (St. Louis, U.S.A)제품을 사용하였다.

나. 단백분해효소 저해제의 정제

명란 250 g에 1 L의 buffer A (25 mM sodium phosphate buffer containing 50 mM NaCl, 1 mM EDTA, 및 1 mM 2-mercaptoethanol, pH 7.0)를 첨가하여 균질화한 후 원심분리 (10,730 × g, 25 min)하여 상층액을 취하였다. 상층액을 20~40% 포화농도의 (NH4)2SO4를 첨가한 다음 상기와 같은 조건에서 원심분리하여 얻은 침전물에 10 mL의 buffer B (25 mM sodium acetate buffer containning 50 mM NaCl, 1 mM EDTA, and 1 mM 2-mercaptoethanol, pH 5.5)를 첨가하여 녹인 다음 같은 용매에 24시간 투석하여 염을 제거하였다. CM-Sepharose column (2.6 × 30.0 cm)에 투석한 시료 20 mL을 loading하여 분획하였다. 단백질은 0~1.0 M NaCl의 농도 내에서 직선구배에 의하여 유속 1 mL/min로 용출하였다. Papain에 대한 저해활성 (inhibitory activity)이 높은 분획물의 최대 peak의 50% 이상 fraction을 모아서 buffer C (25 mM sodium phosphate buffer, 0.15 M NaCl, 1 mM EDTA, and 1 mM 2-mercaptoethanol, pH 7.5)로 투석한 다음 한외여과로 15 mL로 농축하여 Sephacryl HR-100 (Pharmacia Biotech, Uppsala, Sweden) column (2.6 × 90 cm)에 loading 하여 유속 0.5 mL/min으로 용출하였다.

다. 저해활성 (inhibitory activity) 측정

단백분해효소저해제의 저해활성은 Borla et al. (1998) 및 Weerasinghe et al. (1996)의 방법을 수정하여 측정하였다. 즉 시료 200 μL를 80℃에서 10분간 가열하고 buffer C에 녹인 0.1 U papain 용액 100 μL를 가하여 37℃에서 5분간 반응시킨 후 같은 buffer에 녹인 250 μL의 azocasein 용액 (0.32 mg/mL)을 첨가하여 30분간 반응하였다. 20% trichloroacetic acid (TCA) 용액 700 μL를 가하여 반응을 정지한 다음 원심분리 (10,730 × g, 5 min)하여 얻은상층액과 1 N NaOH 용액을 9:10

(v/v)의 비율로 혼합하여 440 nm에서 흡광도를 측정하였으며, 단백분해효소저해제의 저해활성 1 unit (U)은 papain 활성 1 U의 감소로 정의하였다.

라. High Performance Liquid Chromatography (HPLC) purification

Sephacryl S-100 column chromatography로 분리된 peak를 25 mM MgCl2 와 0.2 M NaCl을 함유한 50 mM Tris-HCl buffer (pH 7.2) 로 24시간 투석한 다음 한외여과 (YM-5, Amicon, U.S.A)로 50 μL로 농축하여 HPLC Shodex 803 column (Showa Denco Co., Japan)에 loading 하여 같은 용매를 사용하여 유속 0.5 mL/min로 용출하였다.

마. 온도 및 pH 안정성

단백분해효소 저해제의 온도 및 pH 안정성 측정은 온도 5~80 ℃, pH 4~8의 범위에서 단백분해효소를 30분간 평형화하고 papain 및 azocasein과 혼합하여 37 ℃에서 30분간 반응시킨 다음 papain에 대한 잔존 저해활성 (residual inhibitory activity)을 측정하였다.

바. 분자량 측정 및 순도 검정

정제된 단백분해효소저해제의 분자량은 Laemmli (1970)의 방법에 따라 12% polyacrylamide gel을 사용하여 SDS-PAGE로 측정하였으며 전기영동시킨후 Rm (relative mobility) 값에 따라 표준곡선을 작성하고 정제된 단백분해효소의 Rm 값을 대응시켜 산정하였다. 염색은 Coomassie brilliant blue R-250 (45% methanol, 10% acetic acid, 0.1% Coomassie brilliant blue R-250)용액과 silver stain kit (BioRad Laboratories, Hercules, CA, U.S.A)을 사용하였으며, 45% methanol + 10% acetic acid 혼합용액으로 탈색하였다.

사. 단백질 정량

단백질의 정량은 Bradford (1976)의 방법으로 BioRad protein assay kit (CA, U.S.A.)을 사용하여 측정하였으며 표준단백질은 bovine serum albumin을 사용하였다. 정제과정 중의 단백질의 양은 280 nm에서의 흡광도 값으로 표시하였다.

3. 결과 및 고찰

가. 단백분해효소 저해제의 정제

Ammonium sulfate 농도 20-40%에서 비활성 (specific inhibitory activity)은 1.1 U/mg, yield는 22.7%로 나타났으며 다른 농도의 염석구간에 비하여 가장 높은 단백

분해효소 저해활성을 나타내었다.

Table 1. Purification of protease inhibitor from the eggs of Alaska pollock (Theragra chalcogramma)

Sample	Total protein concentration (mg)	Total inhibitory activity (U)	Specific inhibitory activity (U/mg)	Yield (%)	Purity (fold)
Extract	1,191.7	354.0	0.3	100.0	1.00
Ammonium Sulfate (20~40%)	70.2	80.4	1.1	22.7	3.90
CM Sepharose	6.5	54.0	8.4	15.3	28.20
Sephacryl HR-100					
Peak I	1.1	11.3	10.2	3.2	34.10
Peak II	0.3	4.3	15.6	1.2	52.51

CM-Sepharose chromatography에서 두 개의 peak를 얻었으며 이는 Tsai et al. (1996)이 잉어 (Carp, Cyprinus carpio) 난소에서 CM-TSK chromatography로 분리한 단백분해효소저해제의 peak와 유사한 pattern을 나타내었다.

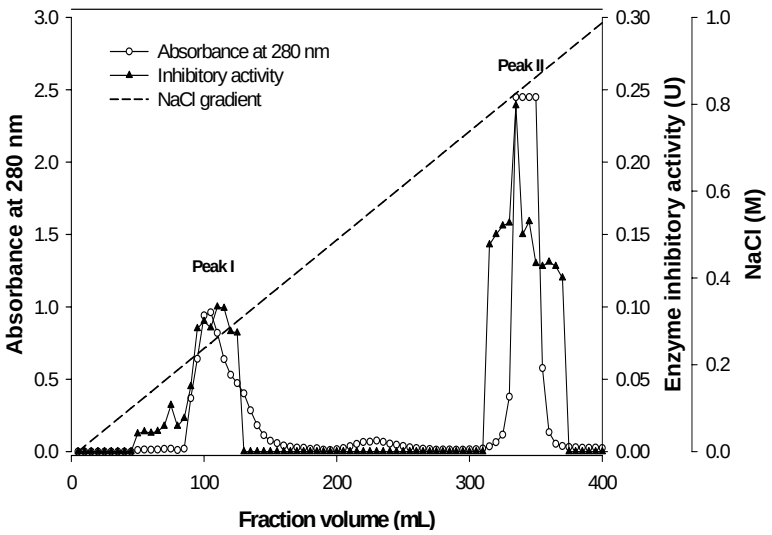

Fig. 1. CM-sepharose chromatographic pattern of ammonium sulfate fraction (20-40%) from the crude extract of the eggs of Alaska pollock (Theragra chalcogramma).

Borla et al. (1998)은 몇 종의 어류에서 단백분해효소저해제를 ion exchange chromatography로 정제할 때 비활성은 1-7 U/mg의 범위라고 보고하였으나 본 실

험에서는 Peak II (elution volume 310-370 mL)에서 8.4 U/mg으로 더 높은 비활성이 측정되었다. Synnes (1998)가 연어 껍질에서 정제한 cysteine 단백분해효소 저해제 또한 ion exchange chromatography에서 두개의 peak를 나타내었으며 두 번째 peak에서 높은 저해활성을 나타내어 본 실험의 결과와 유사하였다. 두 번째 peak중 최대저해활성 (maximal inhibitory activity)의 50% 이상 활성을 가진 fraction을 pooling 한 뒤 buffer C에 투석하여 Sephacryl HR-100 gel filteration chromatography한 결과를 Fig. 2에 나타내었다.

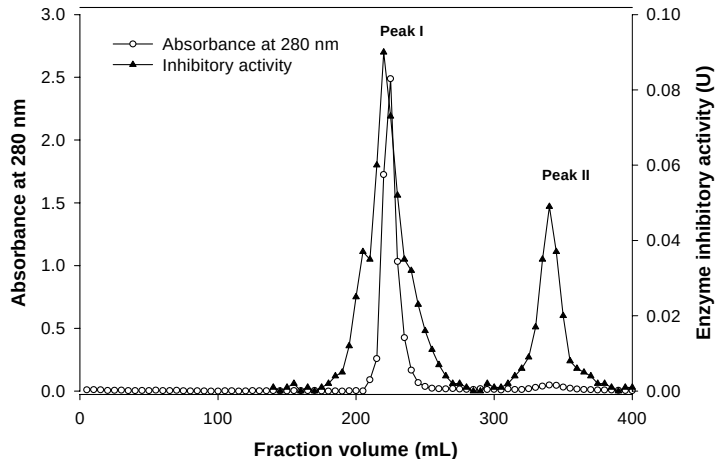

Fig. 2. Sephacryl HR-100 gel filtration chromatographic pattern of Peak II fraction from CM-sepharose chromatography.

Sephacryl gel filteration chromatography로 정제하였을 때 Peak I 및 Peak II의 비활성은 각각 10.2 및 15.6 U/mg, purity는 34.10 및 52.21 folds로 Peak II 가 Peak I 보다 높았으나 수율은 낮았다 (Table 1). Peak I 및 II 모두 연어란의 단백분해효소저해제 (3.8 U/mg 및 66 folds) (Yamashita and Konagaya, 1991)에 비하여 비활성은 높았으나 purity는 낮았으며 수율은 각각 3.2 및 1.2%로 수율에 상당한 문제점이 있었다. 어류의 단백분해효소저해제는 열안정성이 우수하며 (Synnes, 1998), 일반적으로 어란에서 분리·정제한 단백분해효소저해제는 열에 안정하여 정제 과정 중 가열 처리를 통한 어란 내의 내인성 단백분해효소 (endo-protease)의 제거가 용이하다고 하였으나 (Barrett, 1981), 본 실험에서 시료에 잔존하는 내인성 단백분해효소의 실활을 위해 가열한 다음 (80℃, 15 min) 상기와 동일한 실험조건상에서 정제하였을 때 염석에서는 동일한 농도 (20-40%)의 분획물의 저해활성이 가장 높았으나 비활성 및 purification yield는 0.13 U/mg 및 12.6%로 가열하지 않은 시료에 비하여 낮았다 (data not shown). 그러므로 본 실험에서는 가열처리를

하지 않은 시료를 실험에 사용하였다. CM-Sepharose column chromatography에서는 가열한 시료와 가열하지 않은 시료 모두 두 개의 peak를 나타내었으나 (Fig. 3), Sephacryl gel filteration chromatography로 분리하였을 때 가열한 시료는 하나의 peak만을 나타내었고 (Fig. 4), HPLC에서는 두 개의 peak로 잘 분리되지 않았다 (Fig. 5). 시료를 가열하였을 때 (80 ℃, 15 min) 시료속의 단백분해효소저해제는 구형 구조가 선형 구조로 변성된 것으로 생각되며 단백질의 선형 구조는 분자 크기에 의한 분리력을 기초로 하는 gel filteration chromatography 및 HPLC 상에서 peak의 분리가 용이하지 않은 것으로 알려져 있다 (Welling, 1989).

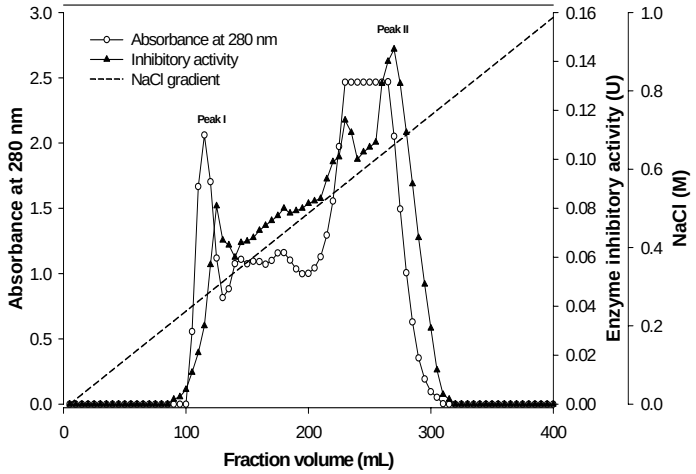

Fig. 3. CM-sepharose chromatographic pattern of ammonium sulfate fraction (20-40%) from the crude extract of the heated eggs of Alaska pollock (Theragra chalcogramma)

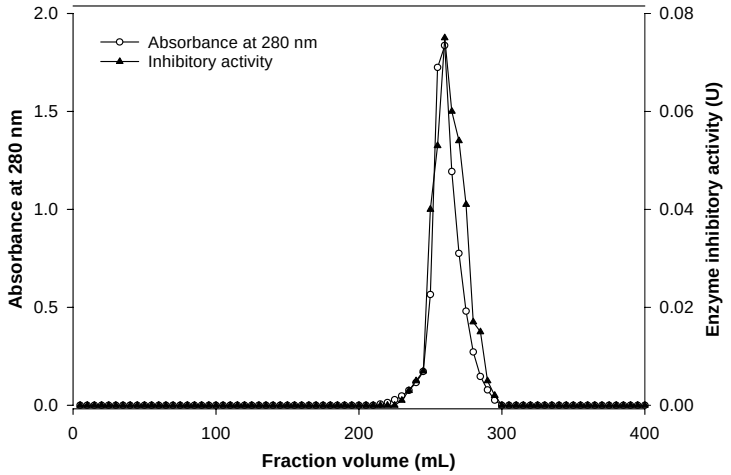

Fig. 4. Sephacryl HR-100 gel filtration chromatographic pattern of Peak II fraction from CM-sepharosechromatograph

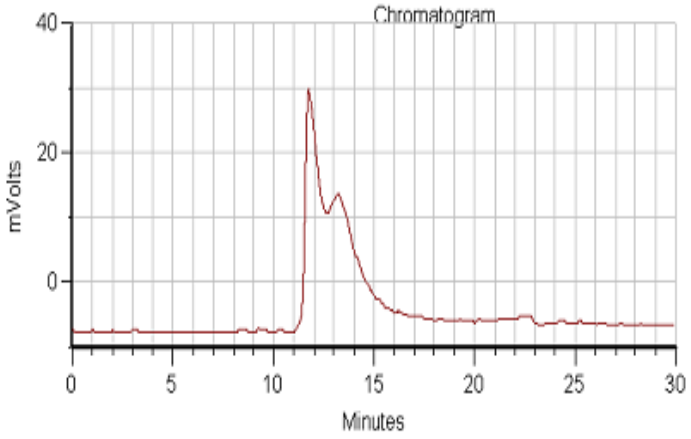

Fig. 5. HPLC pattern of peak fraction from Sephacryl HR-100 gel filtration chromatography.

나. SDS-polyacrylamide gel electrophoresis

Sephacryl gel filteration chromatograph전기영동 한 결과를 Fig. 6에 나타내었다. Peak I은 두개의 protein band를 나타내었고, Sephacryl HR-100 gel filtration chromatography에서 얻은 두개의 활성 Peak (I 및 II)를 얻었고 (not data shown) Peak II는 한 개의 band를 나타내었다.

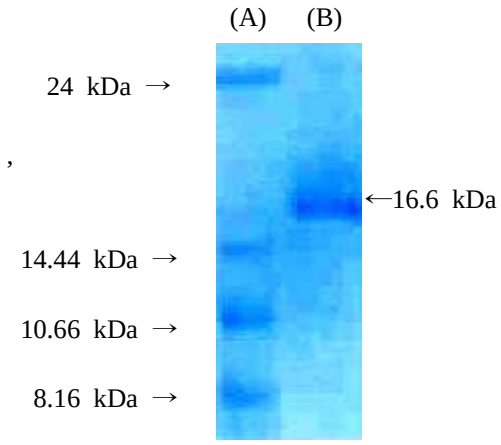

Fig. 6. SDS-Polyacylamide gel electrophoresis of Alaska pollock(Theragrchalcogramma) protease inhibitor. (A) standard marker ; trypsinogen (24 kDa), myoglobin I+II (14.44 kDa), myoglobin I+III, (10.66 kDa), and myoglobin I (8.16) kDa. (B) peak II.

Peak I의 전기영동 결과로 얻어진 2개의 band의 분자량은 각각 66.7 및 16 kDa 이었다. (not data shown) 66.7 kDa band는 Barret (1986)의 cystatin group의 분류 (stefin, cystatins 및 kininogen) 중 kininogen과 분자량이 비슷한 것으로 예상된다

(68-120 kDa). 16 kDa band는 연어란에서 분리한 단백분해효소 저해제 (Yamashita and Konagaya, 1991)의 분자량과 일치하였으며 연어의 뇌, 심장 및 신장에서 분리한 단백분해효소저해제의 분자량은 13 kDa 이었다 (Yamashita and Konagaya, 1996). 또한 잉어란 단백분해효소저해제의 분자량 (12 kDa) (Tsai et al., 1996) 및 난백 cystatin (12 kDa) (Barrett, 1981) 보다는 약간 높았으나 일반적인 cystatin의 분자량 (10-20 kDa) (Abe et al., 1987)의 범위 안에 있었다. Peak II의 분자량은 16.6 kDa으로 Peak II 또한 cystatin으로 판단된다.

다. 온도 및 pH 안정성

명란 단백분해효소저해제의 저해활성은 35 ℃ 이상에서 급격히 감소하기 시작하여 80 ℃에서 최초 저해활성의 약 70%가 감소하였다.

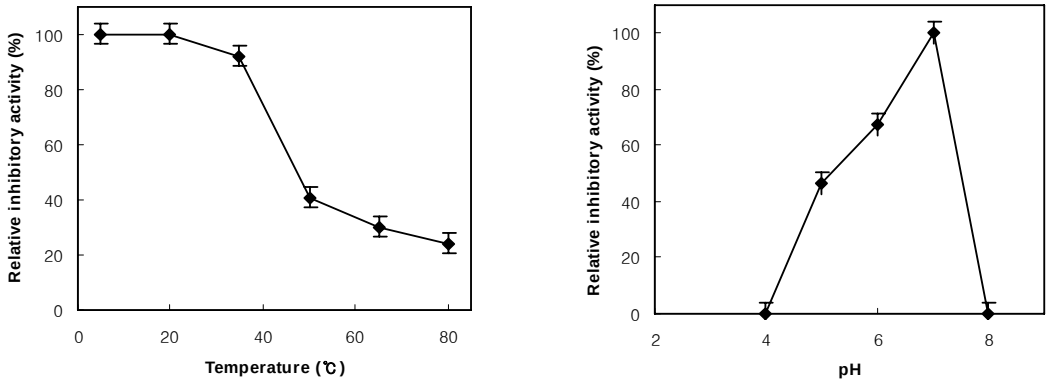

Fig. 7. Effects of temperature and pH on the stability of Alaska pollock (Theragra chalco-gramma) protease inhibitor.

연어 껍질의 단백분해효소저해제는 80 ℃에서 최초 저해활성의 60%가 감소하여 (Synnes, 1998) 본 실험의 결과와 유사하였으며 내열성이 약한 것으로 생각된다. 또한 명란 단백분해효소저해제는 60 ℃에서 30분간 반응하였을 때 잔존 저해활성 (residual inhibitory activity)이 약 35%까지 감소하였다. 수산 연제품의 되풀림 현상 (modori)의 주원인으로 알려져 있는 cathepsin B, H, and L 등과 같은 내인성 단백분해효소는 50-60 ℃에서 안정한 것으로 알려져 있기 때문에 (An et al., 1994), 명란 단백분해효소저해제가 cathepsin류의 단백분해효소를 저해하는 데는 한계가 있을 것으로 생각된다. pH 안정성은 중성부근의 pH에서 안정한 것으로 나타났으며 pH 4 및 8 에서는 저해활성이 나타나지 않았다 (Fig. 7). 어육 내 잔존하는 내인성 단백분해효소인 cathepsin B, H 및 L은 중성 또는 알칼리 pH에서 안정하므로

(Visessanguan et al., 2003) 명란 단백분해효소저해제는 수산연제품의 내인성 단백분해효소저해제의 활성억제를 위해 사용할 수 있을 것으로 생각된다. 연어 이리(soft milt)에서 정제한 단백분해효소저해제의 경우 pH 5-7에서 안정하였으며(Kawabata and Ichishima, 1997), 연어란에서 분리된 단백분해효소저해제는 pH 4 이상에서 저해활성이 감소하기 시작하여 pH 7 이상의 알칼리 pH에서는 저해활성의 약 80% 이상이 감소되는 차이를 보였다 (Yamashita and Konagaya, 1991). 따라서 명란 단백분해효소 저해제를 수산연제품의 품질열화를 억제하기 위한 내인성 단백분해효소저해제의 저해에 사용할 경우 내열성이 약한 문제점이 있을 수 있으나 pH 안정성의 경우 내인성 단백분해효소의 최적 활성 pH인 중성 pH에서 안정하므로 저해효과를 얻을 수 있을 것으로 생각되어지며 추후 온도 및 pH의 복합적인 안정성 실험이 필요할 것으로 생각된다.

라. 단백분해효소저해제의 특성

명란 단백분해효소저해제는 cysteine 단백분해효소인 papain 및 cathepsin L을 저해하는 것으로 나타났으며(Table 2). serine 계 단백분해효소인 trypsin에 대한 저해활성은 없는 것으로 나타났다(data not shown).

Table 2. Inhibitory activity of Alaska pollock egg and egg white protease inhibitor against papain and cathepsin proteases

Inhibitor	Specific inhibitory activity (U/mg)c	
	Papain	Cathepsin L
Alaska pollock egg	15.60 b	29.04 a
Egg white	37.71 b	16.05 b

a,b, Means in the same column with different superscripts are significantly different ($p<0.05$).
c Mean values obtained from four replications.

시판되고 있는 난백저해제 (egg white inhibitor)의 papain 및 cathepsin L에 대한 저해 비활성은 37.71 및 16.05 U/mg로 명란 단백분해효소저해제 (15.60 및 29.04 U/mg)와 비교하였을 때 papain에 대한 저해활성은 높았으나 cathepsin L에 대한 저해활성은 낮았다. Cathepsin 계 단백분해효소는 어육내에 존재하여 Surimi의 gel 연화현상 (modori)에 큰 영향을 미치는 것으로 알려져 있으며 (An et al., 1994) 명란 단백분해효소저해제는 온도 안정성이 약한 약점이 있으나 37℃에서의 저해활성 실험에서 난백 저해제 보다 cathepsin L에 대한 저해활성이 높았으므로 surimi의 modori를 방지에 효과적으로 사용할 수 있을 것으로 사료된다.

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제 2 절 물곰치알 (Glassfish egg)로부터 저분자 단백분해 효소 저해제 (Low-molecular weight protease inhibitor) 정제 및 구조분석

1. Introduction

The changes in functional and organoleptic properties of fish muscle are a consequence of proteolytic activity (An and others 1996; Stoknes and Rustad 1995; Visessanguan and others 2003). Cathepsin was a member of cysteine proteases in which cathepsin B and L were shown to cause softening in chum salmon (Yamashita and Konagaya 1990). Heat stable alkaline protease was hypothesized to responsible for the heat induced softening of surimi gel at near 55 to 60 oC (Hammann and others 1990). Cathepsin L was found to be predominant proteinase involved in heat induced degradation of the myofibrillar protein in Pacific whiting surimi (An and others 1994). It is necessary to improve the functionality of lower grade surimi by adding protease inhibitor such as cystatin which can inhibit the endogenous cysteine proteases (Morrissey and others 1995).

Cystatin is widely distributed in animal tissues and body fluids and divided into three groups on the basis of molecular structure (Li and others 1998). Family I cystatin lacks disulfide bonds, for example, cystatin A (Takahashi and others 2000), cystatin B (Osawa and others 2003), and rat cystatin (Makita and others 1998). Typical family II cystatin contains two disulfide bonds, for example, human cystatin S (Isemura and others 1986), chicken cystatin (Colella and others 1989), mouse cystatin C (Yamaza and others 2001), and rat cystatin A (Takeda and others 1994). Both families are also characterized by molecular

weights between 10 and 20 kDa. Kininogen belongs to family III (Abe and others 1987). Kininogen are single chain glycoproteins containing three cystatin-like domains with molecular weight of 68 to 120 kDa (Barret and others 1986).

Cysteine protease inhibitors, cystatin, were reported to be purified from ovarian fluid of carp (Tsai and others 1996), chum salmon egg (Yamashita and Konagaya 1991), Atlantic salmon and Arctic charr (Olenen and others 2003). The inhibitor in the egg of fishes might take part in the protective role from microorganisms, embryogenesis, and the regulation of early embryonic growth (Burley and Vadehra 1989). Cystatin may also contribute to defense against viral proteases which are required for virus replication (Argos and others 1984).

There is a strong demand to prevent the deterioration of surimi based product or fish meat by inhibiting digestivefish muscle proteases. The most effective way to inhibit the fish muscle proteases is to use natural inhibitor. Therefore, the objective of this study was to purify and identify the protease inhibitor from glassfish egg.

2. Materials and Methods

가. Materials

The eggs were taken from a mature glassfish, Liparis tanakai, immediately after caught and stored at 40 ℃ until used. Papain, trypsin, cathepsin, azocasein, and protein molecular weight marker were purchased from Sigma Chemical Co. (St. Louis, MI, U.S.A.). The other chemicals used in this study were the first reagent grade. Sephacryl HR 100, CM Sepharose, and CNBr-activated Sepharose 4B were purchased from Amersham Pharmacia Biotech, Ltd. (Uppsala, Sweden).

나. Purification of protease inhibitor

250 g of fish egg was homogenized in 1 L of 25 mM sodium phosphate buffer (pH 7.0) containing 50 mM NaCl, 1 mM EDTA, and 1 mM2-mercaptoethanol (buffer A). The cell and tissue debris were precipitated and removed from homogenate by centrifuging at 10,000 g for 25 min. The supernatant was heated at 80 ℃ for 10 min, cooled at room temperature, and centrifuged at same condition as described above. The heated extract was fractionated with ammonium sulfate at 40 to 80% saturation. The precipitated

fraction was dissolved in buffer A and then dialyzed overnight against 50 mM sodium acetate buffer (pH 5.5) containing 50 mM NaCl, 1 mM EDTA, and 1 mM2-mercaptoethanol (buffer B). The dialyzed was applied to CM Sepharose column (2.6 x 30.0 cm) equilibrated with the same buffer in advance. The protease inhibitors were eluted from the column by application of a sodium acetate-buffered (pH 5.5) linear (0 to 1 M) NaCl gradient at flow rate of 1 mL/min. Fractions containing greater than 50% of maximal peak activity were pooled and dialyzed against 25 mM sodium phosphate buffer (pH 7.5) containing 0.15 M NaCl, 1 mM EDTA, and 1 mM2-mercaptoethanol (buffer C). Pooled fractions were concentrated by ultrafiltration (10 kDa cutoff membrane, Amicon Co., Beverly, MA, U.S.A.) and then loaded onto Sephacryl column (2.6 x 90.0 cm) equilibrated with the buffer C. The protease inhibitor was then eluted at flow rate of 0.2 mL/min.

다. Affinity chromatography

7 g of CNBr-activated Sepharose 4B was washed and swelled on glass filter (size G3) with 1.5 L of 1 mM HCl. 20 mL of 5 mg/mL papain solution in coupling buffer, 1 M NaHCO3 (pH 8.3) containing 0.5 MNaCl, was mixed with gel and stirred at 4 ℃ overnight. The gel was transferred to 40 mL of blocking agent, 0.2 M glycine (pH 8.0) and stirred at 4 ℃ overnight. The gel was washed with 0.1 M acetate buffer (pH 4.0) containing 0.5 MNaCl and then with coupling buffer. Finally, the gel was packaged in column (1.0 x 20.0 cm) and equilibrated with buffer A. 50 mL crude extract of fish egg was loaded on affinity chromatography column and the column was washed with buffer A. Protease inhibitor was eluted with 50 mMtrisodium phosphate buffer (pH 10) containing 50 mM NaCl at flow rate of 0.3 mL/min.

라. Inhibitory activity assay

According to the modified method of Borla and others (1998), protease inhibitory activity was determined by measuring the inhibitory degree of papain activity against azocasein as the substrate. 200 μL of 1.7 μg/mL inhibitor solution in buffer A was added to 100μL of papain solution (0.1 U of activity) in buffer A. The inhibitor-papain mixture incubated at 37 ℃ for 5 min was added to 250 μL of 3.2 mg/mL azocasein solution in buffer A. This mixture was

reacted at 37 ℃ for 30 min and then its reaction was stopped by adding 700 μL of 20% trichloroacetic acid (TCA). The control was prepared by substituting 200 μL of inhibitor solution with 200 μL of buffer A. Blank was also prepared by adding 700 μL of 20% TCA in advance before adding 250 μL of substrate solution. 720 μL of supernatant centrifuged at 10,000 g for 5 min was added to 800 μL of 1 N NaOH for exposing the color. Papain activity was expressed as the absorbanceat 440 nm. Inhibitory activity was calculated by difference between papain activities without/with inhibitor. One unit of inhibitory activity was defined as one unit decrease of papain activity.

마. Determination of Ki

0-30 μg/mL inhibitor, 0.5 mg/mL papain, and 0.1-6.4 mg/mL azocasein solution were prepared for determining the kinetic parameters. Km and Vmax values for papain acting on azocasein were calculated by hyperbolic regression analysis of Michaelis-Menten (Michaelis and Menten 1913). Ki was determined using a Dixon plot analysis (Dixon and Webb 1979). Inhibitory activities of glassfish egg inhibitor were measured at three different azocasein concentrations (2, 1, and 0.5 times of Km).

바. Heat and pH stability

Heat stability of the purified inhibitor was determined by incubating the inhibitor preparation at 580 ℃ and pH 4-10 for 30 min. Residual papain inhibitory activity was then determined at 37 ℃ as described above.

사. Electrophoresis

Sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) was done using 12% polyacylamide slab gels at pH 8.3 as described by Laemmli (1970). Purified protease inhibitors in sample buffer (100 mM Tris-HCl buffer containing 2.5% sodium dodecyl sulfate, 0.01% bromophenol blue, 2% glycerol, and 10% -mercaptoethanol, pH 6.8) were heated at 95 oC for 4 min. 5 μL of 1.5 mg/mL protein inhibitor in sample buffer were electrophorized.

아. Amino acid sequence

Protease inhibitor was transferred from 12% SDS-PAGE gel to polyvinylidene difluoride (PVDF) membrane as described by Gravel (2002).Amino

acid sequence was determined using PERKIN 491 protein sequencer (Perkin Elmer Inc., Wellesley, MA, U.S.A.).

자. Synthesis of glassfish egg protease inhibitor

According to the modified method of Salas and others (2004), the glassfish egg protease inhibitor was synthesized by the Fmoc solid-phase method by use of an automated peptide synthesizer (Peptron TM III-R24, Peptron, Daejeon, Korea). After deprotection of the synthesized protease inhibitor from the resin, the peptide was purified and analyzed by reverse-phase HPLC (Waters 2690 Separations Module, Waters, Milford, U.S.A.) using Waters C18 analytical RP column. The glassfish egg protease inhibitor was identified by use of a mass spectrometer (HP 1100 Series LC/MSD, Hewlett-Packard, Roseville, U.S.A.).

차. Protein concentration

Protein concentration was measured according to the manufacture procedure of Bio-Rad protein kit (Bio-Rad Lab. Inc., Hercules, CA, U.S.A.) using bovine serum albumin as the calibration standard. The relative protein content of chromatography fractions was estimated by absorbance at 280 nm.

3. Results and Discussion

가. Purification of protease inhibitor

The conventional purification step of protease inhibitor was summarized in Table 1. Because protease inhibitor was a heat stable, heating treatment is known tobe very effective to eliminate the other enzymes or inhibitors in the fish egg (Barrett 1981). When the heated fish egg extract was precipitated by ammonium sulfate precipitation, the specific activity and purification fold were 0.31 U/mg and 3.38 folds, respectively.

The fractionation pattern of protease inhibitor by CM Sepharose chromatography is shown in Fig. 1.

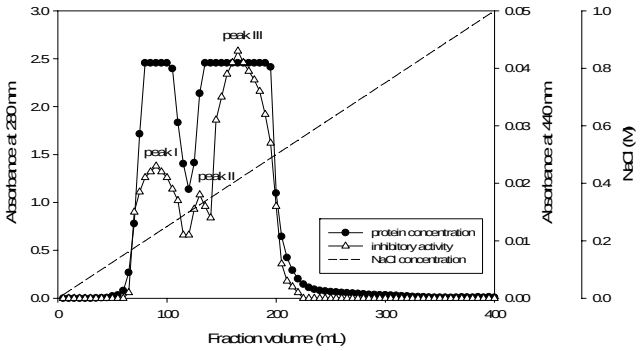

Fig. 1. CM Sepharose chromatography pattern of glassfish egg protease inhibitor.

There were two protein and three inhibitory peaks in which Peak III showed the highest inhibitory activity with 1.41 U/mg of specific activity and 15.36 folds of purification degree (Table 1). Tsai and others (1996) reported that protease inhibitor peak from ovarian carp was eluted lately on CM-TSK chromatography pattern. Borla and others (1998) reported that specific activities of some fish species between 1 and 7 unit/mg were obtained after pooling azocaseinolytic active ion exchange chromatography fractions, which was similar to above results.

Table 1. Purification of the protease inhibitor from glassfish egg by ion exchange and gel permeation chromatography

Purification Step	Total Protein (mg0	Total activity (units)	Spesific activity (units/mg)	Yield (%)	Purity
Heated extract	20,031.00	1,840.00	0.09	100.00	1.00
40-80% $(NH_4)_2SO_4$	1,504.60	465.00	0.31	25.30	3.38
CM Sepharose (Peak III)	29.50	41.50	1.41	2.30	15.36
Sephacryl HR-100 (Peak II)	1.09	1.56	1.43	0.10	15.57

There was a single protein peak on Sephacryl HR 100 chromatography pattern with three papain inhibitory peaks (Peak I, II, and III) (Fig. 2).

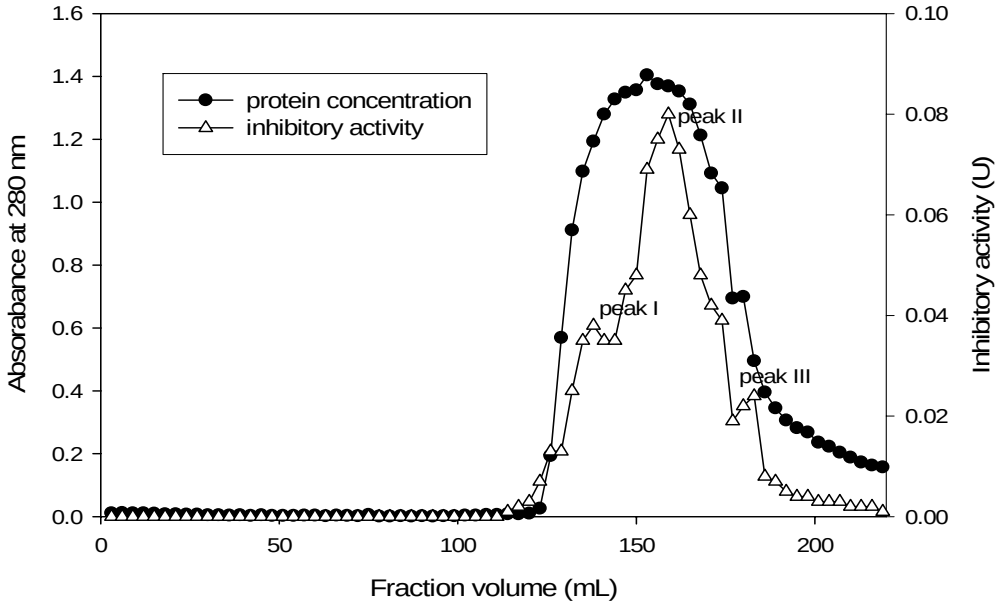

Fig. 2. Sephacryl HR 100 gel chromatography pattern of glassfish egg protease inhibitor.

But on the result of SDS-PAGE, two protein bands were corresponded to highest inhibitory active Peak II, in which their molecular weights were 67 and 18 kDa, respectively (Fig. 3A).

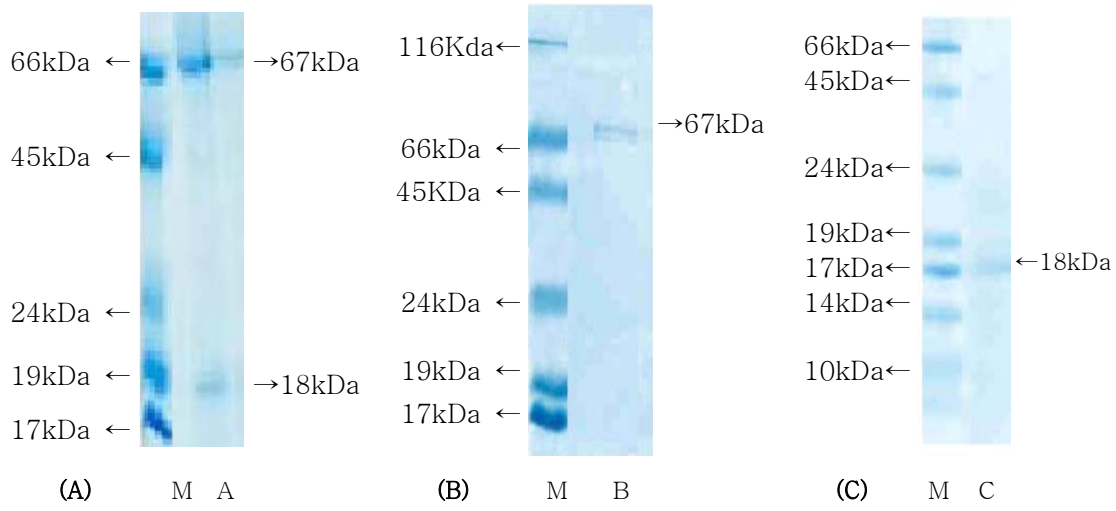

Fig. 3. SDS-PAGE pattern of the purified glassfish egg inhibitor. M, protein marker; A, Peak II of gel permeation chromatography; B, Peak I of affinity chromatograph ; C, Peak II of affinity chromatograph.

Heating treatment in the first step of purification eliminated heat labile protein and increased specific inhibitory activity of extract glassfish egg from 0.08 U/mg (Table 2) to 0.09 U/mg (Table 1).

Table 2. Purification of the protease inhibitor from glassfish egg by affinity chromatography

Purification step	Total protein (mg)	Total inhibitory activity (U)	Specific Inhibitory activity (U/mg)	Yield (%)	Purification (fold)
Egg extract	23,437.50	1,875.00	0.08	100.00	1.00
Affinity chromatography	1.18	4.69	3.97	0.25	49.69

But protein structure might be changed from globular form (tertiary and quaternary structures) to linear form (secondary and primary structures) by heat denaturation. Therefore, two protease inhibitors could not be separated by ion exchange and gel permeation chromatography very effectively. In generally, linear form of polypeptide could not be effectively separated by gel permeation chromatography (Welling and Wester 1989). Otherwise, glassfish egg protease inhibitor is considered to be dimer with big and small molecular weights, thus resulted in single peak on gel permeation chromatography and two bands on SDS-PAGE. In this purification step, specific activity and purification degree of glassfish egg protease inhibitor were 1.43 U/mg and 15.57 folds, respectively (Table 1), which were lower than 3.8 U/mg and 66 folds of chum salmon egg protease inhibitor (Yamashita and Konagaya 1991).

Because of two bands on SDS-PAGE, affinity chromatography, CNBr-activated Sepharose 4B coupled with papain as a ligand, was applied for purifying the protease inhibitor from glassfish egg. There were two protein peaks on affinity chromatography pattern, but only small protein peak showed two inhibitory active peaks (Fig. 4).

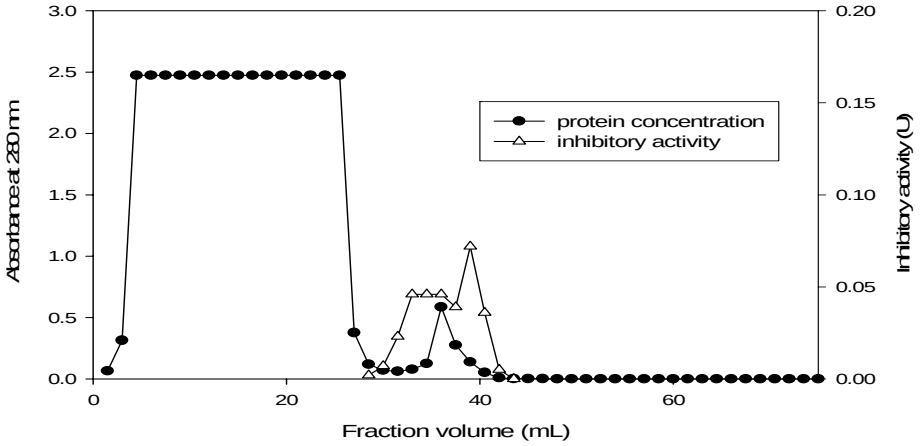

Fig. 4. Affinity chromatography pattern of glassfish egg protease inhibitor.

The big band with MW 67 kDa is corresponded to Peak I (fraction volume 36 mL) in affinity chromatography pattern and the small band with MW 18 kDa is Peak II (fraction volume 39 mL) (Fig. 3BC). Approximately 170 μg of purified protease inhibitor was obtained from 250 mg of glassfish egg. Yield and purity of affinity chromatography were 0.25% and 49.69 folds, respectively (Table 2). Yamashita and Konagaya (1991) isolated protease inhibitor with MW 16 kDa from chum salmon egg in which its purity increased by 78 folds with 1.3% recovery.

CNBr-activated Sepharose 4B coupled papain as an affinity chromatography matrix was effective to purify the protease inhibitor from glassfish egg. It is possible to purify the protease inhibitor by one step purification of affinity chromatography, which resulted in saving the time and labor of purification. On the other hand, the conventional purification procedure of inhibitor by ion exchange and gel permeation chromatography is time-consumingprocedure and results in losing target material. Therefore yield of purified inhibitor with single step purification, 0.25 %, was the higher than 0.10 % of yield with conventional procedure. When glassfish egg extract was loaded on affinity column and then washed with buffer A, protease inhibitors were usually bound on active site of papain-Sepharose 4B matrix. The weak bound inhibitor(67 kDa) was eluted earlier at fraction volume 36 mL than strong bound inhibitor (18 kDa) at fraction volume 39 mL. The protease inhibitor of glassfish egg with small molecular weight (18 kDa) but strong inhibitoryactivity against papain was used

for further experiments in this study.

나. Amino acid sequence

Amino acid sequence of the inhibitor with 18 kDa was N-his-ala-asn-arg-val-met-pro-glu-met-asn-met-glu-tyr-met-glu-ala-C. Among the 17 amino acid residues, there were 9 different kinds of amino acid in which 5 of them were the same amino acids in protease inhibitor from chum salmon (Yamashita and Konagaya 1991). Amino acid sequence of glassfish egg inhibitor with 18 kDa was compared with that of cystatins; human cystatin C (Abrahamson and others 1987), human cystatin S (Turk and Bode 1991), andchicken cystatin (Colella and others 1989). However, no apparent similarity between the sequence of the glassfish inhibitor and the known cystatins was found. Only 3 amino acids were coincided with the other amino acid sequences of cystatins. Because no cysteine residue was found in amino acid sequence, glassfish egg inhibitor was classified into family I which had no disulfide bond. The disulfide bond would be formed by SH group of two or more cysteine residues in inhibitor. Chum salmon egg inhibitor hadcysteine residues which formed disulfide bond and was classified into cystatin family II (Yamashita and Konagaya 1991).

다. Properties of protease inhibitor

The glassfish egg protease inhibitor inhibited papain and cathepsin, cysteine proteases (Table 3), but did not inhibittrypsin, serine protease (data not shown).

Table 3. Comparison of inhibitory activity of glassfish egg protease inhibitor with others against papain and cathepsin proteases

Papain	Cathepsin	
Natural glassfish egg	19.7b	36.84a
Synthesized	16.98b	32.76a
Egg white	37.71a	16.05b
Chymotrypsin potato I	2.00c	4.12c

a,b,c Means in the same column with different superscripts are significantly different ($p < 0.05$).
d Mean values obtained from four replications.

Specific inhibitory activities of natural glassfish egg inhibitor against cathepsin and papain were significantly different in which against cathepsin was

the higher. Animal protease inhibitor is the stronger binding ability to animal protease like cathepsin than plant protease inhibitor like papain.

Comparison of inhibitory activity of glassfish egg with other inhibitors is shown in Table 3. Specific inhibitory activity of glassfish egg and itssynthesized inhibitors against papain were not significantly different. Specific inhibitory activity of glassfish egg inhibitor against papain, 19.97 U/mg, was rather higher than 16.98 U/mg of the synthesized. But both activities were the lower than 37.71U/mg of specific activity of egg white inhibitor. Otherwise, specific inhibitory activities, 36.84 and 32.76 U/mg, of glassfish egg and its synthesized inhibitors against cathepsin, an important protease in fish muscle, were significantly different from and the higher than 16.05 U/mg of egg white inhibitor. Based on the above data, glassfish egg inhibitor was more suitable to use in surimi industry to inhibit the heat stable protease than egg white inhibitor. Glassfish egg and its synthesized protease inhibitors were classified into the group of cystatin family I because of the lack of disulfide bridges. Otherwise, egg white inhibitor with disulfide bridges was classified into cystatin family II (Turk and Bode 1991).

To determine the Ki value of glassfish egg inhibitor on papain, the velocity of azocasein hydrolyzed by papain was measured without/with inhibitor at different azocasein concentrations. Ki was calculatedby Dixon plot, plot of 1/V vs [I] (Dixon and Webb 1979). Glassfish protease inhibitor was noncompetitive inhibitor against papain (Fig. 5) because the X value (inhibitor concentration) of three different linear regressions based on the substrate concentrations was the same, 4.44 nM and Y value (inverse of velocity) was at inverse of Vmax, 11.07 unit-1.

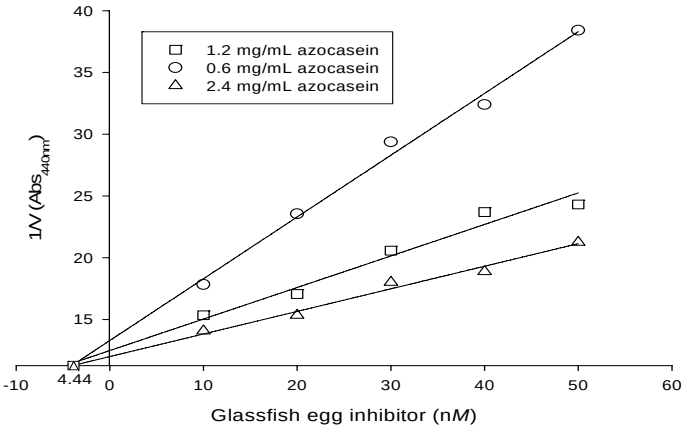

Fig. 5. Dixon plot of papain inhibition with glassfish egg protease inhibitor at different concentrations of azocasein.

The inhibitor constant (Ki) of glassfish egg inhibitor, 4.44 nM, was the higher than 0.1 nM of chum salmon egg inhibitor (Yamashita and Konagaya 1991) and 1.45 nM of tomato cystatin (Wu and Haard 2000), while the lower than 4.70 nM of transgenic tomato cystatin (Jacinto and others 1998) and 6.10 nM of cowpea cystatin (Fernandes and others 1991) (Table 4).

Table 4-Inhibitor constant (Ki) of glassfish proteases inhibitor

Source	Ki(nM)
Glassfish	4.44*
Salmon	0.1 (Yamashita and Konagaya 1991)**
Tomato cystatin	1.45 (Wu and Haard 2000)**
Tomato transgenic cystatin	4.70 (Jacinto and others 1998)*
Cowpea cystatin	6.10 (Fernandes and others 1991)**

* Azocasein as substrate
** 1.4-M benzoyl-DL-arginine-β-naphthylamide (BANA) as a substrate.

In this case, Ki values of chum salmon egg inhibitor, cowpea cystatin, and tomato cystatin were determined using papain as a protease and BANA as a substrate. On the other hand, Ki value of transgenic tomato protease inhibitor was determined using papain as a protease and azocasein as a substrate, which was the same as in our study. Glassfish egg inhibitor with lower Ki value was more effective to inhibit papain than transgenic tomato inhibitor at the same

condition.

The glassfish egg inhibitor was relatively stable at alkaline pH 7-10 with maximal activity at pH 8 (Fig. 6). The residual inhibitory activity after incubation at 37 ℃ and pH 8 for 30 min was 70%. There was no inhibitory activity on papain at acidic condition (pH < 6). Tomato cystatin lost the inhibitory activity by 10% at the pH range of 411 and 4 ℃ for 24 h incubation (Wu and Haard 2000). Alkaline protease inhibitor from Actinomycetes was stable at pH 5-12 and room temperature for 1 h incubation (Pandhare and others 2002). Glassfish egg inhibitor can be used to inhibit cysteine endoproteinasesin surimi gelling process, in which its optimal activity is at neutral and weak alkaline condition. Most of fish muscle cathepsin is also active at neutral and weak alkaline condition (Visessanguan and others 2003).

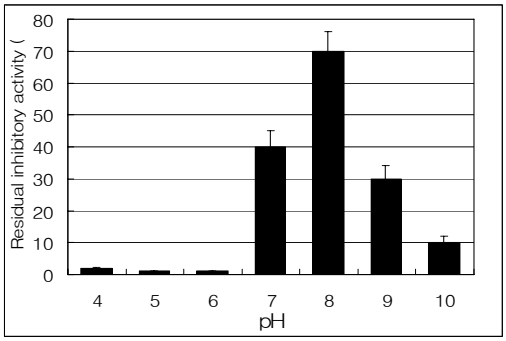

Fig. 6. The stability of glassfish egg protease inhibitor at different pHs.

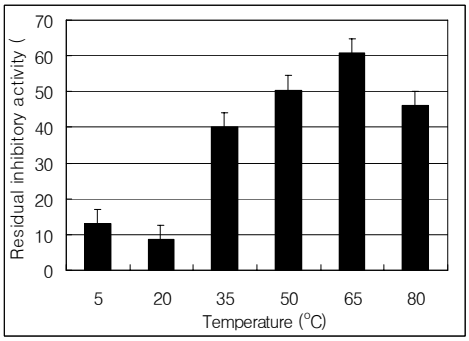

Fig. 7. The stability of glassfish egg protease inhibitor at different temperatures.

The glassfish egg protease inhibitor was more stable at higher temperature (Fig. 7). 60.8 and 40.1% inhibitory activities were retained after incubation at 65 and 80 ℃ for 30 min, respectively. The inhibitory activity of glassfish egg inhibitor was lost by 90% below 20 ℃. The stability of glassfish egg protease inhibitor with 60.8% residual inhibitory activity incubated at 60 ℃ for 30 min was more stable than that with 40% of alkaline protease inhibitor of Actinomycetesat the same condition (Pandhare and others 2002). Glassfish protease inhibitor with 60.8% residual inhibitory activity at 60 ℃ is able to inhibit endogenous fish muscle proteases such as cathepsin B, H, and L with optimal activity at 5060 ℃ (An and others 1994). Based on above results, glassfish protease inhibitor will be one of the most effective ways to inhibit

heated-alkaline protease in fish muscle which causes the deterioration of surimi during gelling process.

4. Conclussions

The molecular weights of glassfish egg protease inhibitors were 18 and 67 kDa, respectively. Those inhibitors were not separated by ion exchange and gel permeation chromatographies, but separated well by CNBr-activated Sepharose 4B chromatography. The yield and purity of 18 kDa protease inhibitor were 0.25% and 49.68 folds, respectively. The amino acid sequence of 18 kDa inhibitor was N-his-ala-asn-arg-val-met-pro-glu-met-asn-met-glu-tyr-met-glu-ala-C. The 44% of amino acids was identical but no apparent homology to cystatin of chum salmon. The inhibitor constant (Ki) of glassfish egg protease inhibitor against papain was 4.44 nM, but its inhibitory activity was stable at even 50-65 ℃ and pH 8. Glassfish egg inhibitor is classified into a cystatin family I of cysteine protease inhibitor because of no cysteine residue for the formation of disulfide bonds.

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제 3 절 물곰치알 (Glassfish egg)로부터 고분자 단백분해 효소 저해제(High-molecular weight protease inhibitor) 정제 및 구조분석

1. Introduction

The inhibitor of cysteine protease was first isolated from chicken egg white in 1968 [30]. This inhibitor was further characterized as cystatin and the first memberof the cystatin superfamily [3]. The cystatin superfamily is divided into three structurally related families; stefins, cystatins, and kininogens [3]. Family 1 (stefin) lacks both disulfide bridges and carbohydrates [3]. Stefin has a molecular mass of around 11 kDa, is the smallest in the cystatin superfamily. This family includes cystatin A and B found in different mammals; human [1, 27], cow [37], rat [34], sheep [28], and pig [20]. Family II (cystatin) is also single chain with one domain protein and about 2 kDa larger than the family I inhibitor. The polypeptide chains of cystatins contain two disulfide bonds near their C-terminus [3]. Family III (kininogen) consists of a N-terminal heavy chain combined with a variable length light chain. The heavy chain has three cystatin-like domains. Based on the length of the light chain [3], the kininogen is divided into two sub-families; a high molecular weight kininogen (HMW kininogen, ~120 kDa) and a low molecular weight kininogen (LMW kininogen, ~68 kDa).

The interaction between proteases and their inhibitors was a target of intensive study for the last two decades. Protease inhibitors were purified from ovarian

fluid carp [36], egg and muscle of chum salmon [40,45], muscle of white croaker [29], Atlantic salmon and Arctic charr [23], and hake, Argentine anchovy, castaneta, rough sead, and sea trout [5]. The specific inhibitors of cysteine proteases are needed in preventing unwanted destructive proteolysis, which can be used in therapy and research [13, 42], toxic for pest [10, 26], and food industry [11, 12].

In industries of surimi-based product, commercial protease inhibitors are used to prevent modori (gel softening) phenomenon and to maximize the gel strength of surimi. The most commonly used inhibitors are bovine plasma protein (BPP), chicken egg white, potato powder, and whey protein concentrate [11, 41]. Because there is some side effect on surimi based product such as change of color [2], fish protease inhibitor is thought to be the best to prevent modori phenomenon.

High molecular weight (HMW) cysteine protease inhibitors were purified from Atlantic salmon (Salmo salar L.) [33], plasma of steelhead trout [7], bowfin [8], Atlantic cod [25], and the sarcopterygian lungfish [21]. HMW cysteine protease inhibitor from Atlantic salmon skin was purified and characterized, and its amino acid sequence was found to be homologous to kininogens [47]. In our previous study, HMW and LMW protease inhibitors were purified from glassfish egg [39]. In the present study, HMW protease inhibitor of glassfish egg was further purified and characterized in order to have basic data for commercialization.

2. MATERIALS AND METHODS

가. Materials

Eggs from a mature glassfish, Liparis tanakai, were harvested immediately after capture and stored at 40 ℃ until used. Papain, trypsin, cathepsin, azocasein, and protein molecular weight marker were purchased from Sigma Chemical Co. (St. Louis, MI, U.S.A.). The remaining chemicals used in this study were the highestreagent grade. Carboximethylated (CM) papain immobilized resin was purchased from Calbiochem-Novabiochem Co. (La Jolla, CA, USA).

나. Purification of Protease Inhibitor

HMW protease inhibitor from glassfish egg was purified by the modified method of Brillard-Bourdet et al. [6]. An 80 g of glassfish egg was homogenized in 240 ml of 50 mM sodium phosphate buffer, pH 6.5, containing 1 mM EDTA (buffer A). Cellsand tissue debris were precipitated and removed from homogenate by centrifugation at 27,390 ×g for 30 min at 4 ℃. The supernatant was loaded onto CM papain immobilized resin column (1.0 10 cm) at a flow rate of 0.3 ml/min and washed with 50 mM sodium phosphate buffer, pH 6.5, containing 0.5 M NaCl and 1 mM EDTA (buffer B). Proteins were eluted from the column with 50 mM K2HPO4/NaOH (pH 11.5) at a flow rate of 1 ml/min. Fractions (1.4 ml each) were collectedin tube containing 0.25 ml of 250 mM KH2PO4, pH 4.5, to bring the pH to neutral.

Because there were two protein bands shown on SDS-PAGE, electro-elution method with GeBA Gel Extraction Kit (Gene Bio Application Ltd., Kfar-Hanagig, Israel) was applied for further purification. After staining, the gel slices containing proteins were excised with a clean sharp scalpel and transferred to GeBAflex-tubes. The tube (midi size) was filled with 800 L of running buffer (0.025 M Tris-base, 0.192 M glycine, and 0.1% SDS), and then closed gently. The tube was placed on the supporting tray in a horizontal electrophoresis tank containing running buffer. Electric current was passed at 100 volt for 85 min. The polarity of the electric current was reversed for 2 min to release the protein from the membrane of tube. Protein suspension was transferred to 1.5 ml micro-tubes and centrifuged at 14,000 g for 1 min to remove gel residues. The purified inhibitor was concentrated by ultrafiltration (cutoff 10 kDa, Millipore Corporation, Bedford, MA, USA).

다. Assay of Inhibitory Activity

(1) Against papain

Glassfish protease inhibitory activity was determined by measuring the degree of inhibition of papain activity using azocasein as the substrate. A 200 l aliquot of 1.7 μg/ml inhibitor solution in buffer A was added to 100 μL of papain solution (0.1 U of activity) in buffer A. The combined solution was incubated at 37 ℃ for 5 min and then added to 250 μL of 3.2 mg/ml azocasein substrate solution in buffer A. Following a 30 min incubation at 37 ℃, the reaction was stopped by adding 700 μL of 20% trichloroacetic acid (TCA). A

control was prepared by substituting 200 μL of inhibitor solution with 200 μL of buffer A. A blank was prepared by adding 700 μL of 20% TCA before adding 250 μL of substrate solution. For color development, the reaction mixture was centrifuged at 10,000 ×g for 5 min, and 720l of the supernatant was added to 800 μL of 1 M NaOH. The absorbance was measured at 440 nm. The inhibitory activity was calculated by the difference between papain activities with and withoutinhibitor. One unit of inhibitory activity was defined as a one unit decrease of papain activity [5].

(2) Against cathepsin L

Inhibitory activity against cathepsin L was measured using Z-Phe-Arg-NMec as the substrate. A 3 ng aliquot of cathepsin L in 500 μL of 0.1% Brij 35 was added to 250 μLof 340 mM sodium acetate buffer (pH 5.5) containing 60 mM acetic acid and 4 mM EDTA, and the mixture was left at 30 ℃ for 1 min to activate cathepsin L. A 1 μL of 3 ng/μL inhibitor solution and 250 μL of 1 mM Z-Phe-Arg-NMec solution in dimethyl sulfoxide were added and then mixed immediately. Following 10 min incubation at 30 ℃, the reaction was stopped by adding 1 ml of 100 mM sodium monocloroacetate (pH 4.3) containing 30 mM sodium acetate and 70 mM acetic acid. Activity of the enzymewith and without inhibitor was determined by measuring the fluorescence of the free aminomethylcoumarin at 370 nm for excitation and at 460 nm for emission. The inhibitory activity was calculated by thedifference between cathepsin activities with and without inhibitor. One unit of inhibitory activity was defined as a one unit decrease of cathepsin L activity [4].

(3) Electrophoresis

The glassfish protease inhibitor was analyzed by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) and polyacrylamide gel electrophoresis (PAGE). Briefly, purified glassfish protease inhibitor was added to sample buffer of SDS-PAGE (100 mM Tris-HCl, pH 6.8, 2.5% SDS, 0.01% bromophenol blue, 2% glycerol, and 10% -mercaptoethanol) to give a final protein concentration of 1.5 mg/ml. After a 4 min incubation at 95 ℃, 5 L of the prepared sample was applied to a 12% SDS-polyacrylamide slab gel at pH 8.3 [17]. Sample buffer of PAGE [40]was composed of 15.5 ml of 1 mM

Tris-HCl buffer, 2.5 ml of 1% bromophenol blue, 7 ml of water, and 25 ml of glycerol, pH 6.8. The further procedure of PAGE was the same as described above.

(4) Thermal and pH Stability

The purified glassfish inhibitor was incubated for 30 min at various temperatures, ranging from 50 ℃ to 80 ℃, and at varying pH values (pH range: 4-10) to determine thermal and pH stability of the protein. Residual papain inhibitory activity was then determined at 37 ℃ as described above.

(5) Inhibitor Constant (Ki)

Aliquots of 0-30 μg/ml glassfish protease inhibitor, 0.5 mg/ml papain, and 0.1-6.4 mg/ml azocasein were prepared for the determination of kinetic parameters. K_m and V_{max} values for papain on azocasein were calculated using the hyperbolic regression analysis of Michaelis-Menten [22]. Ki was determined using a Dixon plot analysis [9]. Theactivity of glassfish egg inhibitor was measured at three azocasein concentrations (2, 1, and 0.5 times Km).

3. RESULTS AND DISCUSSION

가. Purification of Protease Inhibitor

The fractionation pattern of glassfish protease inhibitor by affinity chromatography is shown in Fig. 1.

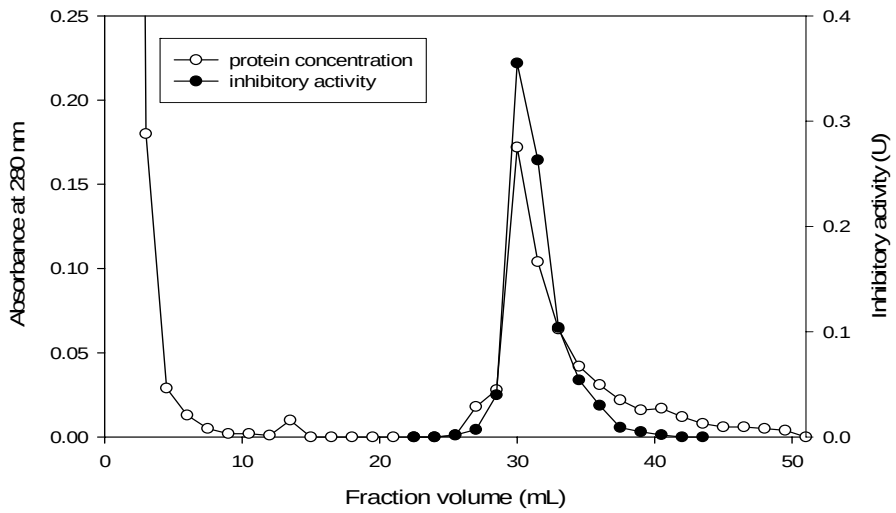

Fig. 1. CM-papain immobolized resin affinity chromatography pattern of HMW protease

inhibitor purified from glassfisg egg.

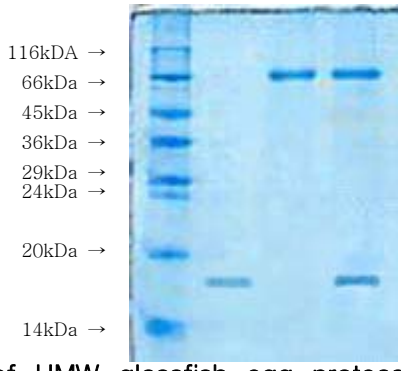

Fig. 2. SDS-PAGE pattern of HMW glassfish egg protease inhibitor. Lane 1. MW protein marker ; Lane 2 and 3. protease inhibitor purified by electro-elution ; Lane 4. protease inhibitor purified by affinity chromatography.

There was one protein peak with high inhibitory activity (8.42 U/mg) against papain. However, on SDS-PAGE, there were two protein bands with MW 67 and 18 kDa (Fig. 2). These two proteins were separated and purified by electro-elution using GeBaflex-tube extraction kit. The specific inhibitory activity and purity of protease inhibitor with MW 67 kDa were 18.57 U/mg and 132.64 fold, respectively. The specific inhibitory activity and purity of protease inhibitor with MW 18 kDa were 19.70 U/mg and 140.71 fold, respectively, a little higher than those of HMW protease inhibitor (Table 1).

Table. 1. Purification of HMW protease inhibitor from glassfish egg.

Purification step		Total protein (mg)	Total inhibitory activity (U)	Specific Inhibitory activity (U/mg)	Yield (%)	Purification (fold)
Egg extract		2555.72	360.00	0.14	100.00	1.00
Affinity chromatography		0.25	2.11	8.42	0.59	60.14
Electro-elution	18 kDa	0.012	0.23	19.70	0.06	140.71
	67 kDa	0.014	0.26	18.57	0.07	132.64

Because of high yield, the higher molecular weight protein (HMW) was selected for further experiments in this study, and its properties were compared with other protease inhibitors. In another study, molecular weight of cysteine protease inhibitor from Atlantic salmon (Salmo salar L) skin was 52 kDa, which

was classified as a kininogen based on its amino acid sequence [47]. Cysteine protease inhibitor with MW 50 kDa was also isolated from the muscle of carp and inhibited both papain and calpain [35]. Family III of cystatin superfamily is divided into two sub-families; high (-120 kDa) and low (-68 kDa) molecular weight kininogens [3]. Therefore, based on its molecular weight, the purified glassfish egg protease inhibitor with MW 67 kDa might be a member of kininogens.

나. Properties of the Protease Inhibitor

Optimal pH and temperature for inhibitory activity of HMW glassfish protease inhibitor were pH 6 (Fig. 3A) and 40 ℃ (Fig. 3B), respectively.

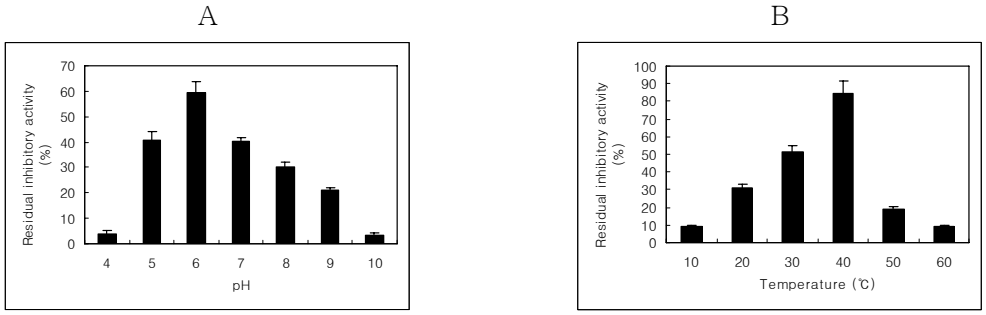

Fig. 3 (A, B). The effect of pHs and temperatures on inhibitory activity of HMW glassfish egg protease inhibitor.

The HMW glassfish egg inhibitor was shown to be relatively stable within a pH range of 5 to 7, with maximal activity at pH 6. The residual inhibitory activity after preincubation at 37 ℃ and pH 6 for 30 min was 85%, but more than 50% of inhibitory activity was lost at extreme pH (< pH 4 and > pH 9) (Fig. 4A).

Other study has shown that human plasma kininogen with MW 83.5 kDa had high inhibitory activity against papain and cruzipain at pH 6.5 [38]. In contrast, LMW glassfish egg inhibitor was shown to be relatively stable within a pH range of 7 to 10, with maximal activity at pH 8 (Fig. 5).

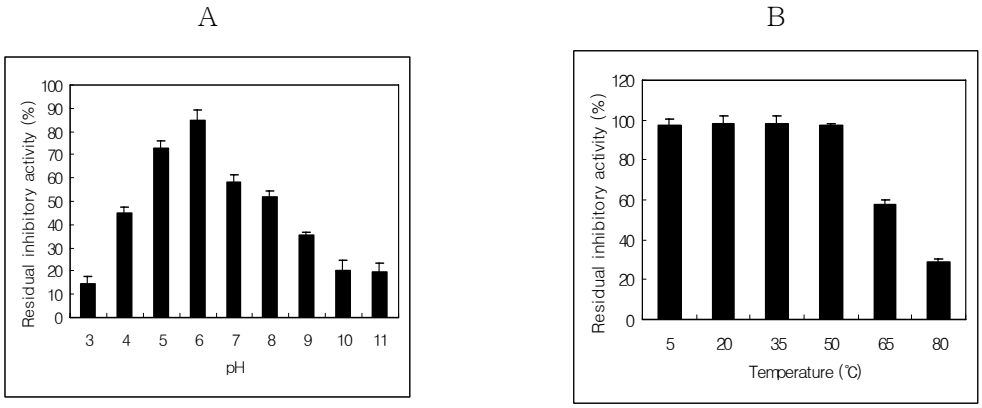

Fig. 4 (A, B). The stability of HMW glassfish egg protease inhibitor at temperature and pHs

Fig. 5. Dixon plot of papain inhibition with HMW glassfish egg protease inhibitor at different concentrations of azocasein.

The residual inhibitory activity after preincubation at 37 ℃ and pH 8 for 30 min was 70%, but no inhibition of papain was observed under acidic conditions (pH < 6) [39].

A raw material of surimi is fresh muscle of fishes. During processing, it will come to be in postmortem stage and decrease in pH of fish muscle or surimi. Because the fish muscle is weakly acidic pH, cysteine-like cathepsin may play an important role in softening gel of surimi before proteases such as alkaline heat stable proteases are active to cleave myosin heavychain and other proteins [18, 24, 19].

The 67 kDa of the glassfish egg protease inhibitor was even more stable at

lower temperatures: Inhibitory activities of more than 90 % were retained after 30 min incubations at 5, 20, 35, and 50 ℃. The inhibitory activity of 70 % was lost when incubated at 80 ℃ (Fig. 4B). In contrast, the LMW glassfish egg protease inhibitor was even more stable at higher temperatures: Its inhibitory activity of 60.8% and 40.1% were retained after 30 min preincubations at 65 ℃ and 80 ℃, respectively [39]. Cathepsin B and L have been found to soften chum salmon [43, 44], tilapia [31], and mackerel [15]. The strength of surimi gel with cathepsin B and L decreased significantly after 2 h incubation at 55 ℃ [16]. Because HMW protease inhibitor from glassfish egg was stable at 50 ℃, this inhibitor might be able to inhibit heat stable cathepsin B and L in surimi gelling process.

To determine the Kivalue of HMW (67 kDa) glassfish egg protease inhibitor with papain, the velocity of azocasein hydrolysis by papain was measured with and without fish egg inhibitor at different azocasein concentrations, and the Ki was calculatedusing a Dixon plot of 1/V vs. [I] [9]. The result showed that HMW glassfish egg protease inhibitor was a competitive inhibitor against papain, as the inhibitor concentration (X value) was the same (97.02 nM) for two different linear regressions based on the substrate concentrations, and Y value (reciprocal of velocity) was at the reciprocal of Vmax, 11.07 unit-1. The Kiof HMW glassfish egg inhibitor (97.02 nM) was higher than the Ki of transgenic tomato cystatin (4.70 nM) [14] and LMW glassfish egg inhibitor (4.70 nM) [39], and was lower than that of the human kininogen (170 nM) [32]. The Ki value of human kininogen was determined using cathepsin B with the fluorogenic peptide substrate, whereas the Ki value of the transgenic tomato cystatin and LMW glassfish egg inhibitor were determined using papain and azocasein as substrates. As HMW glassfish egg inhibitor has a higher Ki, it would be less effective than the transgenic tomato inhibitor to inhibit papain at the same assay conditions.

As shown in Table 2, the HMW glassfish egg protease inhibitor was compared with the activity of chicken egg white cystatin.

Table 2. Comparison of inhibitory activity of HMW protease inhibitory from glassfish egg and chicken egg white against and cathepsin

Protease inhibitor	Relative inhibitory activity(%)d	
	Papain	Cathepsin L
HMW glassfisg egg	98.09a	26.47b
Chicken egg white	97.13a	15.69c

a,b,c Means in the same column with different superscripts are signficantly different($p<0.05$).
d Mean values obtined from three replications.

The HMW glassfish egg protease inhibitor was able to inhibit papain and cathepsin L. The relative inhibitory activity of the glassfish egg protease inhibitors against cathepsin L (26.47%) was significantly higher than chicken egg white protease inihibitor, 15.69%. Otherwise, there was no significantly difference between inhibitory activities of two inhibitors against papain. In a recent study, the LMW glassfish egg inhibitor against cathepsin L (36.84 U/mg) was also significantly higher than that of egg white inhibitor against cathepsin L (16.05 U/mg) [39]. Therefore, both of the glassfish egg protease inhibitors might be used to substitute commercial chicken egg white inhibitor to prevent "modori" phenomenon in surimi-based products.

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제 4 절 연어알 (salmon egg) 단백분해효소저해제의 정제 및 특성

1. Introduction

The cysteine protease inhibitors, whichare widely distributed in animal muscle and body fluid, are classified into three families based on their structural complexities (1). Family I cystatins lack disulfide bonds and include cystatins A (2) and B (3), and rat cystatin β (4). Family II cystatins are characterized by two disulfide bonds and include human cystatin S (5), chicken egg white cystatin (6), mouse cystatin (7), and rat cystatin (4). Molecular weights of Family I and II cystatins range from 10 to 20 kDa. Family III cystatins include kininogens, which are single-chain glycoproteins containing three cystatin-like domains with molecular weights ranging from 68 to 120 kDa (8). Protease inhibitors of the fish eggs are thought to be involved in the protection of eggs from microorganisms, embryogenesis, and the regulation of early embryonic

growth. Cystatins may also contribute to the defense against viral proteases that are necessary for virus replication (9). In addition, cysteine protease inhibitors have also been used for medical treatment of parasite diseases (10) and malaria (11), as well as prevention of modori phenomenon,in which endogenous protease causes deterioration of the gel quality of surimi-based product (12). Some food-grade protease inhibitors such as egg white, bovine plasma protein, potato powder, and whey protein have beenused to prevent modori phenomenon (13) however, they cause side effects such as color changes in surimi-based products (12). Cysteine protease inhibitors were purified from ovarian fluid carp (14), egg and muscle of chum salmon caught inPacific Ocean near Japan (9,15), muscle of white croaker (16), and Atlantic salmon and Arctic charr (17). There is a strong demand to prevent the quality deterioration of surimi-based product or fish meat (18). One of the most efficient methods is to inhibit digestive fish muscle proteases. Therefore, the objective of this study was to purify and characterize the protease inhibitor from the eggs of chum salmon, and to develop a novel inhibitor preventing modori phenomenon in surimi-based products.

2. Materials and Methods

가. Materials

The eggs from a mature chum salmon, Oncorhynchus keta, were harvested immediately after catchingin the coast of East Sea, Korea and were stored at 40 ℃ until used. Papain, trypsin, cathepsin, azocasein, and protein molecular weight markers were purchased from Sigma Chemical Co. (St. Louis, MO, USA). The remaining chemicals used in this study were of first reagent grade. CM Sepharose and CNBr-activated Sepharose 4B were purchased from Amersham Pharmacia Biotech, Ltd. (Uppsala, Sweden).

나. Purification of protease inhibitor

Purification of protease inhibitor was purified by the modified method of Moon and Kim (19). Fish eggs (250 g) were homogenized in 1 L of 25 mM sodium phosphate buffer, pH 7.0, containing 50 mM NaCl, 1 mM EDTA, and 1 mM 2-mercaptoethanol (buffer A). Cell and tissue debris were precipitated and removed from the homogenate by centrifugation at 10,730 ×g for 25 min. To

further purify the extract, the supernatant was dialyzed overnight against 50 mM sodium acetate buffer, pH 5.5, containing 50 mM NaCl, 1 mM EDTA, and 1 mM 2-mercaptoethanol (buffer B). The dialyzed fraction was applied to a CM-Sepharose column (2.6 x 30.0 cm) equilibrated with buffer B. Proteins were eluted from the column using a linear gradient of 0 to 1 M NaCl in sodium acetate buffer (pH 5.5) at 1 mL/min. Fractions containing higher than 50% of maximal inhibitory activity were pooled and dialyzed against a 25 mM sodium phosphate buffer, pH 7.5, containing 0.15 M NaCl, 1 mM EDTA, and 1 mM 2-mercaptoethanol (buffer C). The pooled fractions were concentrated by ultrafiltration using a 10 kDa cutoff membrane (Amicon Co., Beverly, MA, USA). The concentrate was then loaded onto a Sephacryl column (2.6 x 60.0 cm) equilibrated with buffer C and was eluted at 0.2 mL/min.

다. Affinity chromatography

Aliquot (7 g)of CNBr-activated Sepharose 4B was washed and re-swelled on a glass filter (size G3) with 1.5 L of 1 mM HCl. Subsequently, 20 mL of 5 mg/mL papain solution in coupling buffer (1 M NaHCO3, pH 8.3, 0.5 M NaCl) was mixed with the gel and stirred overnight at 4 ℃. The gel was thentransferred to 40 mL blocking agent (0.2 M glycine, pH 8.0) and stirred overnight at 4 ℃. The gel was sequentially washed with 0.1 M acetate buffer (pH 4.0, 0.5 M NaCl) and the coupling buffer. Finally, the gel was poured into a column (1.0 x 20.0 cm), which was then equilibrated with buffer A. Aliquot (50 mL) of the pooled fractions purified by Sephacryl gel permeation chromatography was loaded onto the affinity column and washed with buffer A. The protease inhibitor was eluted with 50 mM trisodium phosphate buffer, pH 10, containing 50 mM NaCl at 0.3 mL/min.

라. Inhibitory activity assay

Chum salmon protease inhibitory activity was determined by measuring the degree of inhibition of papain activity using azocasein as the substrate. Aliquot (200 L) of 1.7 μg/mL inhibitor solution in buffer A was added to 100 μL papain solution (0.1 U activity) in buffer A. The combined solution was incubated at 37 ℃ for 5 min and added to 250 μL of 3.2 mg/mL azocasein substrate solution in buffer A. Following 30 min incubation at 37 ℃, the reaction was stopped by

adding 700 μL of 20% trichloroacetic acid (TCA). A control was prepared by substituting 200 μL inhibitor solution with 200 μL buffer A. A blank was prepared by adding 700 μL of 20% TCA before adding 250 μL substrate solution. For color development, the reaction mixture was centrifuged at 10,000 g for 5 min, and 720 L supernatant was added to 800 μL of 1 N NaOH. The absorbance was measured at 440 nm. Inhibitory activity was calculated based on the difference between papain activities with and without inhibitor. One unit each of the papain and inhibitory activities were defined as the amount of 0.1 M azocasein hydrolyzed per min and one unit decrease of papain activity, respectively (13,20).

마. Heat and pH stability

The purified chum salmon inhibitor was incubated for 30 min at temperatures ranging from 5 to 80 ℃ and at varying pH values (pH range: 2-8) to determine the heat and pH stabilities of the protein. Residual papain inhibitory activity was then determined at 37 ℃ as described above.

바. Electrophoresis

The chum salmon protease inhibitor was analyzed using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and polyacrylamide gel electrophoresis (PAGE, native gel). Briefly, purified chum salmon protease inhibitor was added to the sample buffer of SDS-PAGE (100 mM Tris-HCl, pH 6.8, 2.5% SDS, 0.01% bromophenol blue, 2% glycerol, and 10% -mercaptoethanol) to give a final protein concentration of 1.5 mg/mL. After 4 min incubation at 95oC, 5 L prepared sample was applied to a 12% SDS-polyacrylamide slab gel at pH 8.3 (21). Sample buffer of PAGE (22) was composed of15.5 mL of 1 mM Tris-HCl buffer, 2.5 mL of 1% bromophenol blue, 7 mL water, and 25 mL glycerol, pH 6.8. Mixture of the sample buffer of PAGE and chum salmon protease inhibitor at a final protein concentration of 1.5 mg/mL was applied to a 12% polyacrylamide slab gel at pH 8.3 (21).

사. Protein assay

Protein assay was performed using a protein kit (Bio-Rad Lab. Inc., Hercules, CA, USA) according to the manufacturer's instructions. Bovine serum

albumin was used as the calibration standard. The relative protein concentration of the chromatographically separated fraction was estimated by measuring absorbance at 280 nm.

3. Results and Discussion

가. Purification of protease inhibitor

The purification of chum salmon egg protease inhibitor is summarized in Table 1. The fractionation pattern of protease inhibitor determined by CM Sepharose chromatography is shown in Fig. 1.

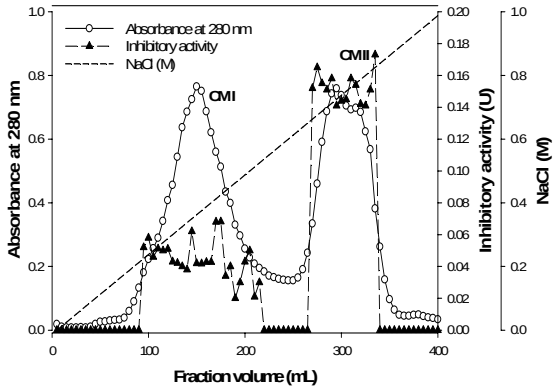


Fig. 1. CM Sepharose chromatography pattern of chum salmon egg protease inhibitor.

Two protein peaks (CM I and CM II) showing inhibitory activity were observed, withthe inhibitory activity of the second peak (CM II) being higher than the first one (CM I).

A similar pattern was obtained from an ovarian carp-isolated protease inhibitor through CM-TSK chromatography (14). Specific inhibitory activities between 1 and 7 U/mg were observed in the pooled azocaseinolytic-active ion exchange chromatography fractions of the protease inhibitors isolated from different fish species (20), which were comparable to the results of this study. Synnes (23) reported that ion exchange chromatography of Atlantic salmon skin protease inhibitor showed two peaks, in which the second peak had higher inhibitory activity. Therefore, the second peak (CM II) of CM-Sepharose chromatography was chosen for further purification by Sephacryl gel permeation chromatography only one protein inhibitory peak was detected, with two protein

bands on SDS-PAGE (data not shown). Therefore, pool of Sephacryl chromatography peak was further fractionated by affinity chromatography, and, among the two protein peaks (elution volumes, 72 and 117 mL) had no inhibitory activity obtained, only the smallest peak showed inhibitory activity against papain (Fig. 2).

The specific inhibitory activity and purification fold of the smallest peak were 4.67 U/mg and 58.11, respectively (Table 1), higher than the specific inhibitory activity of protease inhibitor (3.8 U/mg) of from chum salmon caught in Japan (9).

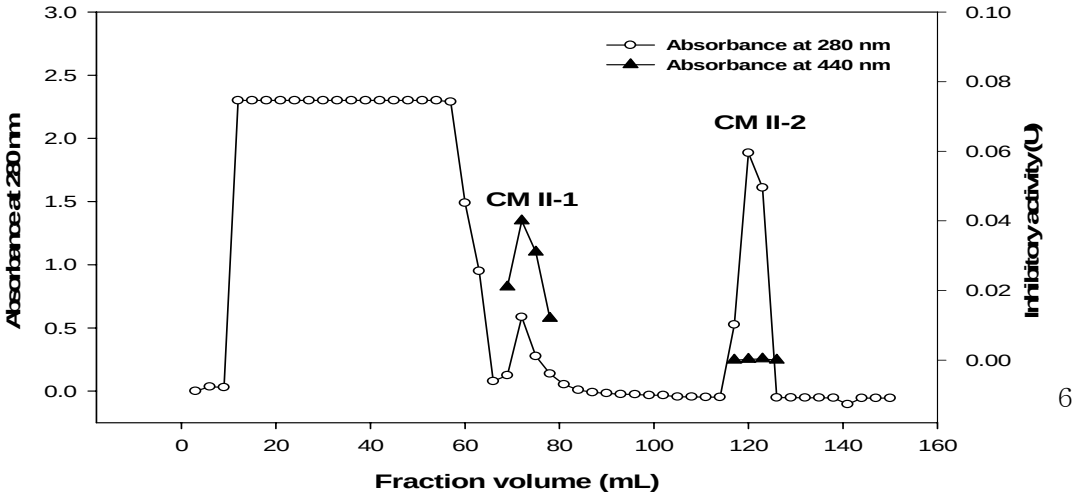


Fig. 2. CNBr-Sepharose 4B-coupled papain affinity pattern of chum salmon egg protease inhibitor.

Table 1. Purification of cysteine protease inhibitor from chum salmon egg by ion exchange and CNBr-Sepharose 4B-coupled papain chromatography

Purification step	Total protein (mg)	Total inhibitory activity (U)	Specific Inhibitory activity (U/mg)	Yield (%)	Purity (fold)
Extract	1,046.00	84.00	0.08	100.00	1.00
CM-Sepharose	5.72	15.60	2.02	13.75	15.14
CMBr-sepharose 4B	0.27	1.26	4.67	1.50	58.11

However, the purification yield and fold (1.5% and 58.11, respectively) of protease inhibitor from chum salmon egg captured in the coast of East Sea, Korea of this study were lower than 1.9% and 93 fold of salmon caught in the coast of the Pacific Ocean, Japan (9). Purification of Atlantic salmon skin protease inhibitor showed similar result on affinity chromatography in that the first peak has higher inhibitory activity than the second one (23).

나. Electrophoresis

SDS-PAGE and PAGE (native gel) analyses of the smallest peak are shown in Figs. 3 and 4, respectively.

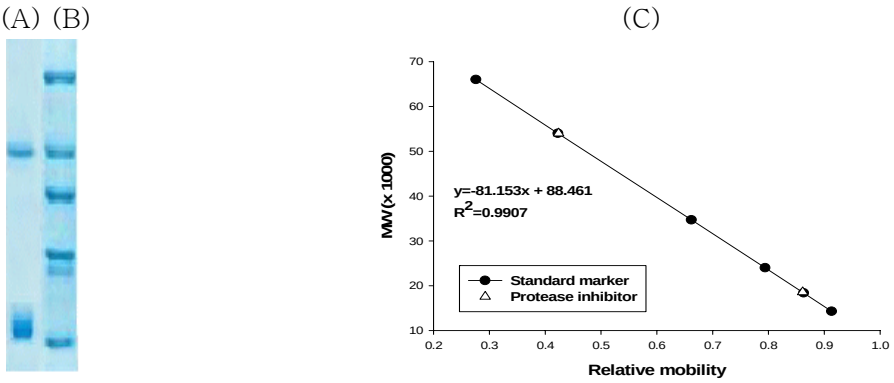
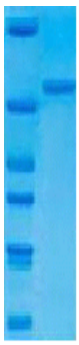


Fig. 3. SDS-polyacylamide gel electrophoresis of chum salmon protease inhibitor. (A), SDS-PAGE of chum salmon egg protease inhibitor(54kDA and 18.6kDa) ; (B), Standard marker : bovine serume albumin(66kDa), egg albumin(54kDa), β-lactoglobumin(18kDa), lysozyme(14.3kDa) ; (C), Estimation of MW of protease inhibitor ; (● : Standard marker, △ : CMⅡ-1).

(A) (B) C



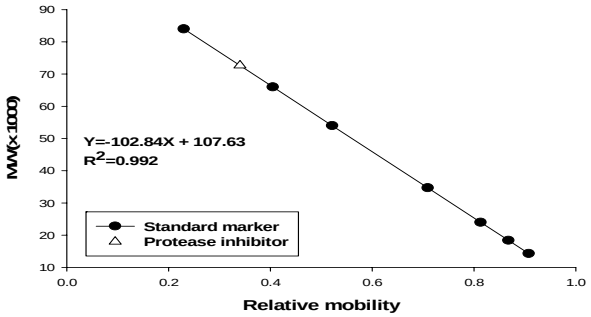


Fig. 4. Polyacylamide gel electrophoresis of chum salmon protease inhibitor. (A), (B), Standard marker : bovine serume albumin (84kDa), pepsin (34.7kDa), trypsinogen (24kDa), β-lactoglobumin 18kDa), lysozyme (14.3kDa) ; (B), chum salmon egg protease inhibitor (72.6kDa) ; (C), Estimation of MW of protease inhibitor.

SDS-PAGE analysis showed two protein bands with MW 54 and 18.6 kDa, whereas PAGE analysis showed only one protein band with MW 72.6 kDa, indicating that chum salmon egg protease inhibitor isa heterodimer protein. SDS-PAGE analysis of the protease inhibitor purified from chum salmon caught in Japan resulted in two protein bands with MW 11 and 16 kDa classified as cystatin (9). Synnes (23) reported that MW of protease inhibitor from Atlantic salmon (Salmon salar L) skin was 76 kDa and classified it as a kininogen (24,25). Kininogn, a single chain glycoprotein consisting of two chains, heavy and light, connected by a single disulfide bond, is easily converted into two-chain forms (26). Therefore, based on these findings, cysteine protease inhibitor from salmon egg with MW 72.6 kDa was tentatively classified as a kininogen.

다. Properties of the protease inhibitor

The chum salmon egg protease inhibitor inhibitedthe cysteine proteases such as papain, and cathepsin (Table 2), but not trypsin, a serine protease (data not shown).

Table 2. Comparison of inhibitory activity of chum salmon egg protease inhibitor with chicken egg white and chymotrypsin potato I against papain and cathepsin L.

Inhibitor	specific inhibitory activity(U/mg)d	
	papain	cathepsin L
Salmon egg	4.67c	28.02a
Chicken egg white	37.71a	16.05b
Chymotrypsin potato I	2.00c	4.12c

a,b,c Means in the same column with different superscripts are signficantly different($p<0.05$).
d Mean values obtined from three replications.

Furthermore, it demonstrated higher inhibitory activity against cathepsin than against papain, possibly because the cathepsin is a fish muscle protease, while papain is a plant protease (9). Although protease inhibitor from chum salmon caught in Japan inhibited papain and cathepsin L, the specific inhibitory activity against papain, 3.8 U/mg (9), was lower than 4.67 U/mg of protease inhibitor from chum salmon caught in Korea in this study (Table 1).

The chum salmon egg protease inhibitor was relatively stable at low temperatures (Fig. 5).

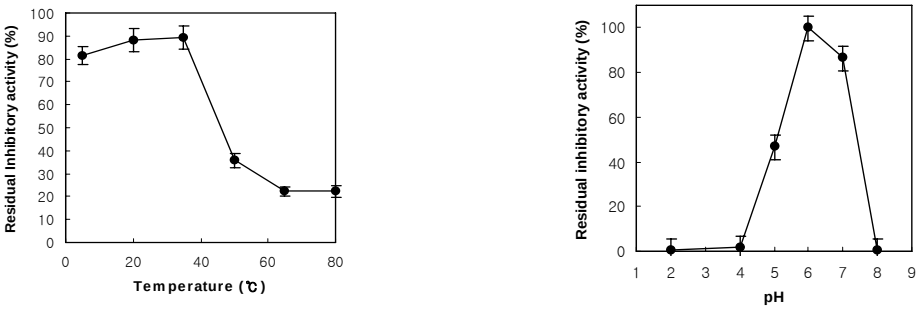


Fig. 5. Effect of temperature and pH on the stability of chum salmon egg protease inhibitor.

Inhibitory activity of 80% was retained after 30 min incubation at 35 ℃. Inhibitory activity of chum salmon egg inhibitor decreased by 60 and 70% when incubated at 50 and 65 ℃, respectively. Although most of the residual inhibitory activity of salmon skin inhibitor remained after incubation at 70 ℃ for 30 min, 60% residual inhibitory activity was lost after incubation at 80 ℃ for 30 min (23). Forty percent residual inhibitory activity of Actinomycetes protease inhibitor remained after incubation at 60 ℃ for 30 min (27). Moreover, chum

salmon protease inhibitor at 60 ℃ was shown to inhibit endogenous fish muscle proteases such as cathepsins B, H, and L, with optimal activity at 50 - 60 ℃ (12).

The chum salmon egg inhibitor was relatively stable within a pH range of 6 to 7, with maximal stability at pH 6 (Fig. 5). The residual inhibitory activity after incubation at 37 ℃ and pH 7 for 30 min was 86.4% however, no inhibition of papain was observed under extreme acid (pH< 4) and alkaline (pH > 8) conditions, as similarly observed by other research group, who showed that protease inhibitor from milt chum salmon was relatively stable within a pH range 5 to 7, and no inhibition of papain was observed under extreme acid and alkaline conditions (28). On the other hand, protease inhibitor from chum salmon caught in Japan was relatively stable within a pH range of 2 to 7, with maximal activity at pH 3. Most endogenous cysteine proteases in fish muscle are also active at weak acid pH, with significant activity at around pH 7 (29).

Therefore, salmon egg protease inhibitor with MW 72.6 kDa in this study could better inhibit cysteine protease in fish muscle and surimi product than that from chum salmon caught in Japan (9). The inhibitory activity of chum salmon egg inhibitor was compared with those of other inhibitors (Table 2). The specific inhibitory activity of chum salmon egg protease inhibitor against cathepsin (28.02 U/mg) was higher than against papain (4.67 U/mg). In contrast, the egg white inhibitor, which is also a member of the cystatain family II (25), demonstrated lower inhibitory activity against cathepsin (16.05 U/mg) than against papain (37.71 U/mg) (Table 2). The specific inhibitory activity of chymotrypsin potato, which is a member of the serine protease inhibitor, against papain and cathepsin were 2.00 and 4.12 U/mg, respectively. Cathepsin, an endogenous protease in fish muscle, plays an important role in softening surimi gel induced by heating (modori phenomenon). Therefore, the chum salmon egg protease inhibitor could be used as a substitute for chicken egg white, which is presently the most commonly used commercial inhibitor, to prevent modori phenomenon in the surimi-based product.

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제 5 절 기타 어종 단백분해효소저해제 정제 및 특성

1. Introduction

Proteolytic enzymes are distributed in all types of organisms including fishes (Barrett, 1994; Stoknes and Rustad, 1995). They catalyze the cleavage of the peptide bond. Proteolytic enzymes can be assigned to four distinct groups on the basis of their catalytic types serine, cysteine, aspartic, and metallo proteases(Kenny, 1999). The cysteine protease is the largest group and includes lysosomal cathepsins. There are many reports on cysteine proteases in muscle of several fish species in which most of them are thermostable and alkaline stable (Folco et al., 1984; Busconi et al., 1984; Lin and Lanier, 1980). The alkaline proteases have an important role in protein metabolism, turnover, post mortem modifications of fish muscle protein (Boye et al., 1980; Wasson, 1992; Ladrat et al., 2004), and softening gel of surimi-based products (Hammann et al., 1990)

The action of proteases was regulated and inhibited by endogenous inhibitor. The interaction between proteases and their inhibitor was a target of intensive studying for the last two decades. Protease inhibitors were purified from ovarian fluid carp (Tsai et al., 1996), and egg and muscle of chum salmon (Yamashita and Konagaya, 1991ab), muscle of white croaker (Sangorrin et al., 2001), and Atlantic salmon and Arctic charr (Olenen et al., 2003).Protease inhibitor in muscle of white croaker, hake, Argentine anchovy, castaneta, rough sead, and sea trout were investigated and compared (Borla et al., 1998).

The specific inhibitors of cysteine proteases are needed in preventing unwanted destructive proteolysis which can be used in therapy and research (Hernandez and Roush, 2002; Pol and Bjork, 2003), toxic for pest (Gruden et al., 1998; Rogelj et al., 2000), and food industry (Hammann et al., 1990; Pandhere et al., 2000). Cysteine protease inhibitors were subdivided into 4 major families: stefin, cystatin, kininogen, and phytocystatin according to occurrence, sequence, and structural similarity (Rassam and Laing, 2004). The stefin is the smallest inhibitor with MW 11 kDa and no difulfide bridges. The cystatin family is 13

kDa with disulfide bridges. The kininogen family is the largest inhibitor with molecular weight of68 to 120 kDa. Phytocystatin family from botanical seed is more homologous to the cystatin family in amino acid sequence, while the lack of disulfide bridgeson these inhibitor is similar to the stefin (Tseng et al., 2002). A cystatin homologous to mammalian cystatin C was first isolated,sequenced from pituitary gland of chum salmon (Koide and Noso, 1994), cloned and expressed (Yamashita and Konagaya, 1996). In this study, protease inhibitor was purified from eggs of different fish species and then compared their specific inhibitory activity against papain. In addition, their stability against temperature and pH was also investigated.

2. Materials and Methods

가. Raw materials

The eggs of five different fishes in Table 1 were taken immediately after caught from November to December in 2002 in Korea and stored at 40 oC until used.

Table 1. Biological classification of fishes studied.

Class			Osteichthyes		
Subclass			Actinopterygii		
Infraclass			Teleostei		
Super order		Clupeomorpha			
Order	Scorpaenifor-mes	Cluperiformes	Gadiformes	Salmoniformes	Osmeriformes
Suborder	Scorpeinoidei	Clupeoidei	Gadeoidei	Salmoniformes	Osmereoidei
Family	Liparidae	Clupeidae	Gadidae	Salmonidae	Osmeridae
Genus Species	Liparis tanakai Glassfish	Clupea pallasii Herring	Theragra Chalcograma Pollock	Oncorhynchus keta Chum salmon	Hypomesus olidus Pond smelt

Papain, trypsin, cathepsin, azocasein, and protein molecular weight marker were purchased from Sigma Chemical Co. (St. Louis, MI, U.S.A.). The other chemicals used in this study were the first reagent grade. Sephacryl HR 100, CM Sepharose, and CNBr-activated Sepharose 4B were purchased from

Amersham Pharmacia Biotech, Ltd. (Uppsala, Sweden).

나. Purification of protease inhibitor

Protease inhibitor of Alaska pollock egg was purified by conventional purification protocol (Tsai et al., 1986). 250 g of fish egg was homogenized in 1 L of 25 mM sodium phosphate buffer (pH 7.0) containing 50 mM NaCl, 1 mM EDTA, and 1 mM 2-mercaptoethanol (buffer A). The cell and tissue debris were precipitated and removed from homogenate by centrifuging at 10,000 g for 25 min. The egg extract was fractionated with ammonium sulfate. The precipitated fraction was dissolved in buffer A and then dialyzed overnight against 50 mM sodium acetate buffer (pH 5.5) containing 50 mM NaCl, 1 mM EDTA, and 1 mM 2-mercaptoethanol (buffer B). The dialyzed was applied to CM Sepharose column (2.6 x 30.0 cm) equilibrated with buffer B in advance. The protease inhibitors were eluted from the column by application of a sodium acetate-buffered (pH 5.5) linear (0 to 1 M) NaCl gradient at flow rate of 1 mL/min. Fractions containing greater than 50% of maximal peak activity were pooled and dialyzed against 25 mM sodium phosphate buffer (pH 7.5) containing 0.15 M NaCl, 1 mM EDTA, and 1 mM 2-mercaptoethanol (buffer C). Pooled fractions were concentrated by ultrafiltration (10 kDa cutoff membrane, Amicon Co., Beverly, MA, U.S.A.) and then loaded onto Sephacryl column (2.6 x 90.0 cm) equilibrated with the buffer C. The protease inhibitor was then eluted at flow rate of 0.2 mL/min.

The protease inhibitors from the glassfish, Pacific herring, chum salmon, and pond smelt eggs were purified by affinity chromatography. 7 g of CNBr-activated Sepharose 4B was washed and reswelled on glass filter (size G3) with 1.5 L of 1 mMHCl. 20 mL of 5 mg/mL papain solution in coupling buffer, 1 M NaHCO3 (pH 8.3) containing 0.5 M NaCl, was mixed with gel and stirred at 4 ℃ overnight. The gel was transferred to 40 mL of blocking agent, 0.2 M glycine (pH 8.0) and stirred at 4 ℃ overnight. The gel was washed with 0.1 M acetate buffer (pH 4.0) containing 0.5 MNaCl and then with coupling buffer. Finally, the gel was packaged in column (1.0 × 20.0 cm) and equilibrated with buffer A. 50 mL crude extract of fish egg was loaded on affinity chromatography column and the column was washed with buffer A. Protease inihibitor was eluted with 50 mM trisodium phosphate buffer (pH 10) containing

50 mM NaCl at flow rate of 0.3 mL/min.

다. Inhibitory activity assay

According to the modified method of Borla et al. (1998), protease inhibitory activity was determined by measuring the inhibitory degree of papain activity on azocasein as the substrate. 200 μL of 1.7 μg/mL inhibitor solution in buffer A was added to 100 μL of papain solution (0.1 U of activity) in buffer A. The inhibitor-papain mixture incubated at 37 ℃ for 5 min was added to 250 μL of 3.2 mg/mL azocasein solution in buffer A. This mixture was reacted at 37 ℃ for 30 min and then its reaction was stopped by adding 700 μL of 20% trichloroacetic acid (TCA). The control was prepared by substituting 200 μL of inhibitor solution with 200 μL of buffer A. Blank was also prepared by adding 700 μL of 20% TCA in advance before adding 250 μL of substrate solution. 720 μL of supernatant centrifuged at 10,000 ×g for 5 min was added to 800 μL of 1 N NaOH for exposing the color. Papain activity was expressed as the absorbance at 440 nm. Inhibitory activity was calculated by difference between papain
activity without/with inhibitor. One unit of inhibitory activity was defined as one unit decrease of papain activity.

라. Thermal and pH stabilities

Thermal stability of the purified inhibitor was determined by incubating the inhibitor preparations at 580 ℃ and pH 4-10 for 30 min. Residual papain inhibitory activity was then determined at 37 ℃ as described above.

마. Inhibitor constant (Ki)

0-30 μg/mL inhibitor, 0.5 mg/mL papain, and 0.1-6.4 mg/mL azocasein solution were prepared for determining the kinetic parameters. Km and Vmax values for papain acting on azocasein were calculated by hyperbolic regression analysis of Michaelis-Menten (Michaelis and Menten, 1913). Ki was determined using a Dixon plot analysis (Dixon and Webb, 1979). Inhibitory activities of glassfish egg inhibitor were measured at three different azocasein concentrations (2, 1, and 0.5 times of Km).

바. Electrophoresis

Sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) was done using 12% polyacylamide slab gels at pH 8.3 as described by Laemmli (1970). Purified protease inhibitors in sample buffer (100 mM Tris-HCl buffer containing 2.5% sodium dodecyl sulfate, 0.01% bromophenol blue, 2% glycerol, and 10% β-mercaptoethanol, pH 6.8) were heated at 95 ℃ for 4 min. 5 μL of 1.5 mg/mL protein inhibitor in sample buffer were electrophorized. β-galactosidase (116 kDa), bovine albumin (66 kDa), egg albumin (45 kDa), trypsinogen (24 kDa), -lactoglobulin (18.4 kDa), myoglobulin from horse heart (16.6), lysozyme (14.3 kDa), myoglobulin I+III (10.66 kDa), and myoglobulin I (8.16 kDa) from Sigma Chemical Co. (St. Louis, MI, U.S.A.) were used as molecular markers.

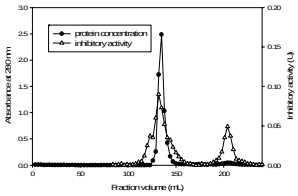
사. Protein concentration

Protein concentration was measured according to the manufacture procedure of Bio-Rad protein kit (Bio-Rad Lab. Inc., Hercules, CA, U.S.A.) using bovine serum albumin as the calibration standard. The relative protein content of chromatography fractions was estimated by absorbance at 280 nm.

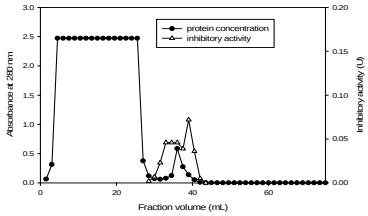
3. Results and Discussion

가. Purification of protease inhibitor

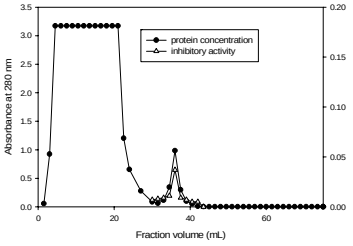
The fraction pattern of Alaska pollock protease inhibitor at final step purification by gel permeation chromatography was shown in Fig. 1. There were two protein peaks with inhibitory activity against papain. The specific inhibitory activity and purity degree of Peak II, 15.6 U/mg and 52.00 folds (Table 2), were higher than 3.41 U/mg and 11.37 folds of Peak I (data not shown). SDS-PAGE of Peak I showed two protein bands with MW 66.7 and 16.8 kDa, respectively (data not shown). Otherwise, SDS-PAGE of Peak II showed one protein band with MW 16.8 kDa (Fig. 2) which was confirmed as a purified Alaska pollock egg inhibitor. The specific inhibitory activity of purified Alaska pollockegg inhibitor, 15.6 U/mg, was higher than 4.67 U/mg of salmon egg inhibitor, but lower than 18.63 U/mg and 19.70 U/mg of Pacific herring and glassfish egg inhibitor, respectively (Table 2).



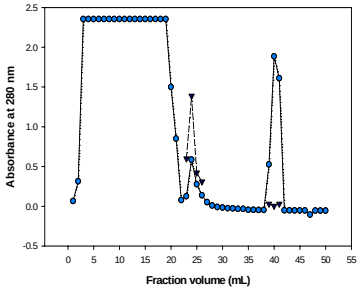
A. Alaska pollock



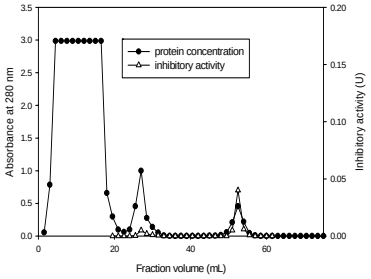
B. Glassfish



C. Pond smelt



D. Chum salmon



E. Pacific herring

Fig. 1. Chromatography pattern of fish egg protease inhibitor. A: on gel permeation chromatogram B, C, D, and E: on affinity chromatogram

Table 2. Inhibitory activity of purified fishes egg inhibitor.

Fish species	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Yield (%)	Purity (fold)
Glassfish	0.15	2.87	19.70	0.25	164.70
Pacific herring	0.17	3.10	18.63	0.17	82.41
Alaska pollack	0.19	3.00	15.60	0.85	52.00
Salmon	0.27	1.26	4.67	1.50	58.11
Pond smelt	0.61	1.90	3.12	0.11	14.46

CNBr-activated Sepharose 4B coupled with papain as a affinity chromatography was used for purifying the protease inhibitor from glassfish egg. There were two protein peaks on affinity chromatography pattern, but only small protein peak showed two inhibitory active peaks (Fig. 1). The protein band with MW 67 kDa (Fig. 2) is Peak I (fraction volume 36 mL) in affinity chromatography

pattern and the protein band with MW 17 kDa (Fig. 2) is Peak II (fraction volume 39 mL). The specific inhibitory activity and purity degree of Peak II, 19.7 U/mg and 164.7 folds (Table 2), were higherthan 3.33 U/mg and 27.87 folds of Peak I (data not shown).

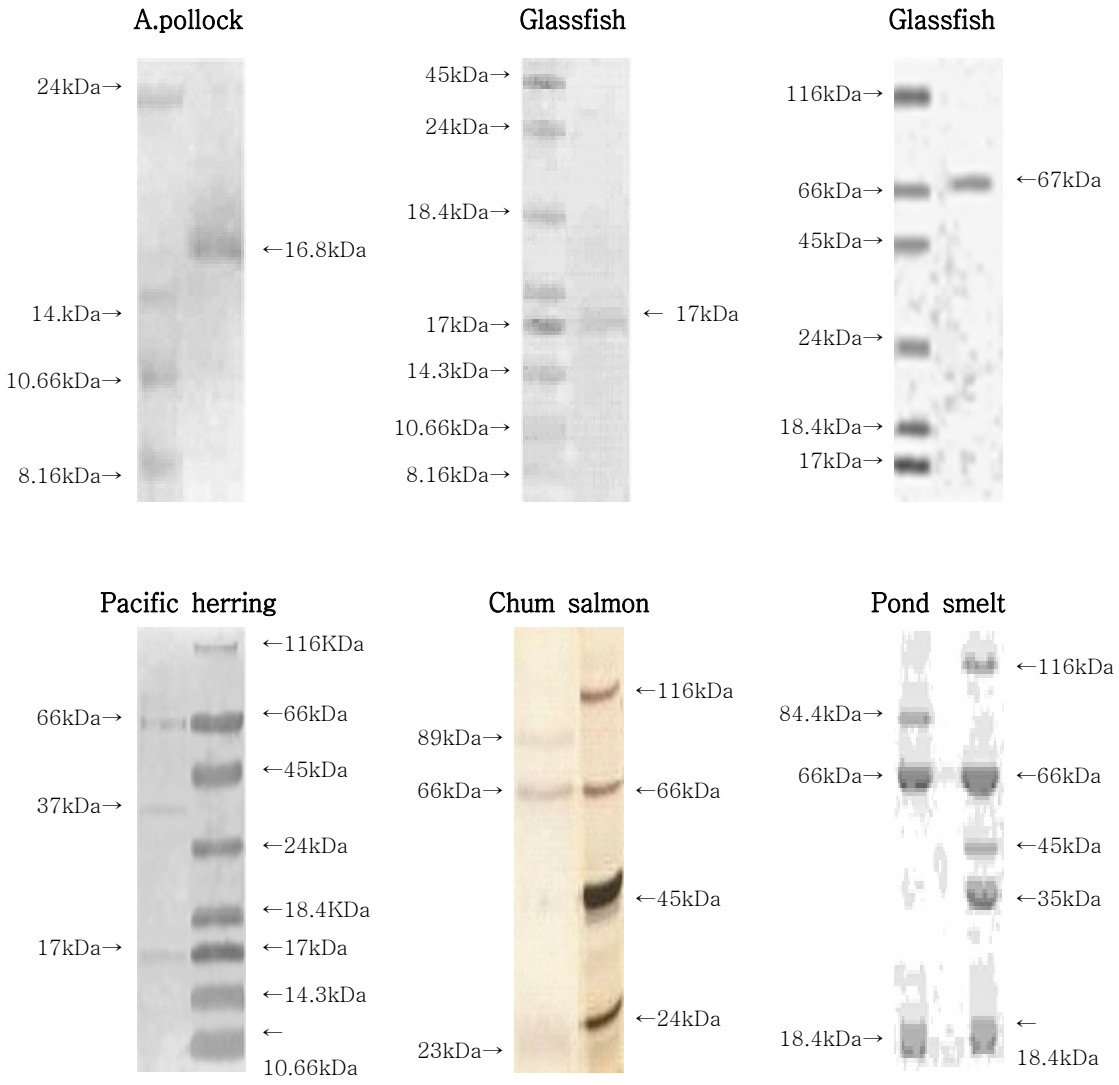


Fig. 2. SDS-PAGE pattern of fish egg protease inhibitors. M was protein marker; I was protease inhibitor; SDS-PAGE pattern of chum salmon was stained by silver stain; The others was stained by coomassie blue.

When pond smelt egg protease inhibitor was purified by affinity chromatography, the elution pattern was similar to that of glassfish egg in which two protein peaks were shown on affinity chromatography pattern. But only small protein peak showed inhibitory active peak (Fig. 1). SDS-PAGE of

the highest inhibitory activity fraction of pond smelt showed three protein bands with MW 84.4, 66, and 18.4 kDa, respectively (Fig. 2). Protease inhibitor with MW 83 kDa purified from human plasma was composed of two high and low molecular subunits held together with a disulfide bond (Ohkubo, et al., 1988). Family 3 of cystatin supermafily was divided to be two subclass high and low molecular weight kininogens. Disulfide bond between domain cystatins of kininogen was usually released due to β-mercaptoethanol in sample buffer and by heating during preparation (Turk, et al., 1986; Auerswald, et al., 1993). Therefore, the purified pond smelt egg protease inhibitor with MW 84.4 kDa might be denaturated by SDS and most was separated to two cystatin domains with MW66 and 18.4 kDa on SDS-PAGE. This inhibitor was considered to be classified to multicystatin which contains two or more cystatin domains. The specific inhibitory activity and purity degree of pond smelt egg protease inhibitor were 3.12 U/mg and 14.46 folds, respectively (Table 2).

Salmon egg protease inhibitor was also purified by affinity chromatography. There were three protein peaks, but only the smallest protein peak had inhibitory activity against papain (Fig. 1). The specific inhibitory activity of the smallest peak was 4.67 U/mg (Table 2). SDS-PAGE of the smallest peak showed three protein bands with MW 89, 66, and 23 kDa, respectively (Fig. 2). According to the mentioned above, the protein band with MW 89 kDa might be also reduced and separated to two cystatin domains with MW 66 and 23 kDa on SDS-PAGE.

Affinity chromatography pattern of Pacific herring egg protease inhibitor was similar to that of salmon egg. There were three protein peaks in which the only Peak II had inhibitory activity against papain (Fig. 1.). The specific inhibitory activity of the Peak II was 18.63 U/mg (Table 2). SDS-PAGE of Peak II showed three protein bands with MW66 , 37, and 17 kDa, respectively (Fig. 2). In this case, Pacific herring egg protease inhibitor with MW 120 kDa might be a kininogen domains 3 (Auerswald, et al., 1993) in which its disulfide was released and separated to three cystatin domains with MW66, 37, and 17 kDa on SDS-PAGE.

The different characteristics of the purified fish egg protease inhibitors may be caused by the phylogenetic origins of fishes (Table 1). The cystatin

superfamily has been subdivided into three families based on their sequence homology, the presence and position of intrachain disulfide bond, and the molecular weight of the protein (Turk and Bode, 1991; Margis et al., 1998). The molecular weights, 16.8 and 17 kDa, of Alaska pollock and glassfish egg inhibitors were presumably classified to cystatin Family 1 or 2. Both families are characterized by molecular weight range from 10 to 20 kDa (Abe et al., 1987). The molecular weight, 120, 89, and 84.4 kDa, of Pacific herring, chum salmon, and pond smelt inhibitors were reasonably classified to cystatin Family 3. Kininogen, Family 3 of cystatin, is characterized by molecular weight of 68 to 120 kDa (Vray et al., 2002).

나. Comparison of inhibitory activity

The inhibitory activity of fish egg protease inhibitors against papain, a member of cysteine protease, was shown in Table 2. The specific inhibitory activity of glassfish egg inhibitor was higher than the others. The specific inhibitory activities of glassfish, Pacific herring, Alaska Pollack, salmon, and pond smelt egg protease inhibitors were 19.70, 18.63, 15.60, 4.67, and 3.12 unit/mg, respectively. The different inhibitory activities of fish egg inhibitors may be due to different of their binding site and amino acid sequence which were depended on their genetic codons (Cleland et al.,1996; Cohen and Hearst, 1996). The genetic codons of their protease inhibitor were different due to the genetically different origin of fishes in this study (Table 1).

다. Comparison of stability

The stabilities of fish egg protease inhibitors at different temperatures and pH values were shown in Table 3.

Table 3. Stability of fish egg protease inhibitors

Inhibitor	Temperature (oC)	pH
Glassfish egg	50-65	8.0
Alaska pollock	5-35	7.0
Chum salmon	5-40	6.0-7.0

Glassfish egg protease inhibitor was stable at 50-65 ℃ and pH 8.0 with 60

and 70% of residual inhibitory activity, respectively. Alaska pollock egg protease inhibitor was stable at 5-35 ℃ and pH 7.0 with 80 and 60% of residual inhibitory activity, respectively, and chum salmon egg protease inhibitor was stable at 5-40 ℃ and pH 6.0~7.0 with 80 and 25% of residual inhibitory activity, respectively. Based on the above data, glassfish egg inhibitor was better than the others to inhibit cysteine proteases in surimi gelling process because its optimal activity is at neutral to weak alkaline condition and 50-70 ℃ which was also optimal condition for modori phenomenon in surimi based product. Most of fish muscle cathepsins are active at neutral and weak alkaline condition (Visessanguan et al., 2003) and 50-60 ℃ (An et al., 1994).

라. Inhibitory activity against proteases

The glassfish egg protease inhibitor inhibited papain and cathepsin, cysteine proteases (Table 4), but did not inhibited trypsin, serine protease (Table 5).

Table 4. Comparison of inhibitory activity of glassfish egg protease inhibitor with others against papain and cathepsin proteases.

Inhibitor	Specific inhibitory activity (U/mg) d	
	Papain	Cathepsin
Glassfish egg	19.7 ^{b}	36.84 ^{a}
Egg white	37.71 ^{a}	16.05 ^{b}
Chymotrypsin potato I	2.00 ^{c}	4.12 ^{c}

a,b,c Means in the same column with different superscripts are significantly different ($p < 0.05$).
d Mean values obtained from four replications.

Table 5. Inhibitory activity of glassfish egg protease inhibitor against papain and cathepsin

Enzymes	Relative inhibitory activity (%)
Papain	16.95 3.50
Trypsin	-2.65 3.05

Specific inhibitory activities of glassfish egg inhibitor against cathepsin and papain were significantly different in which inhibitory activity against cathepsin

was higher than papain. Specific inhibitory activity of glassfish egg inhibitor against cathepsin, 36.84 U/mg, was higher than 16.05 U/mg of egg white inhibitor. Animal protease inhibitor is considered to have stronger binding ability to animal protease like cathepsin than plant protease inhibitor like papain. Glassfish egg inhibitor was therefore very effective to be used in surimi industry to inhibit the heat stable protease than egg white inhibitor.

마. Inhibitor constant (Ki)

In order to determine the Ki value of glassfish egg inhibitor on papain, the velocity of azocasein hydrolyzed by papain was measured without/with inhibitor at different azocasein concentrations. Ki was calculated by Dixon plot, plot of 1/V vs [I] (Dixon and Webb, 1979). Glassfish protease inhibitor was noncompetitive inhibitor against papain because the inhibitor concentration of three different linear regressions based on the substrate concentrations was the same at inverse of maximal velocity (Vmax), 11.07 unit-1. The inhibitor constant (Ki) of glassfish egg inhibitor, 4.44 nM, was lower than 4.70 nM of transgenic tomato cystatin (Table 6).

Table 6. Inhibitor constant (Ki) of glassfish egg protease inhibitor.

Source	Ki (nM)
Glassfish egg	4,44
Tomato transgenic cystatin	4,70 (Jacinto et al., 1998)

Ki value of transgenic tomato protease inhibitor was determined using papain as a protease and azocasein as a substrate (Jacinto et al., 1998), which was the same as in this study.

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제 6 절 재조합 *E. coli*로부터 물곰치 단백분해효소저해제 정제

1. Introduction

Surimi, minced and washed fish muscle consisting of salt-soluble myofibrillar

protein, is widely used as a functional ingredient seafood analogs. Surimi forms thermo-irreversible gels upon heating. Its characteristics of texture are expressed in terms of gel strength, which is the primary determinant for surimi quality and price (An et al., 1996). One of the quality factors affecting certain surimi types is the presence of two major groups, lysosomal cathepsin (Jiang et al., 1997) and the heat stable alkaline protease (Boye and Lanier, 1988). This causes textural degradation of surimi at around 60 ℃ (Hamann et al., 1990).

Protease was classified to four distinct groups on the basis of their catalytic types: serine, cysteine, aspartic, and metallo proteases (Barrett et al., 1994). The names of the enzyme groups refer to the components of their active site. Cysteine protease is a large group including lysosomal cathepsins. Physiologically cysteine proteases have an important role in protein metabolism and turnover. In addition, prohormones, proenzymes, and peptides are activated by cysteine proteases.

Cathepsin, a member of endogenous cysteine protease, is found in several fish species such as Atlantic menhaden (Choi et al., 1999), Pacific whiting (An et al., 1994), chum salmon (Yamashita and Konagaya, 1990a), Carp (Makinodan et al., 1987), Atlantic croaker (Lin and Lanier, 1980), and arrow tooth flounder (Visessanguan et al., 2003). Cathepsin may take a role in softening the gel of surimi based product. Therefore, it is necessary to improve the functionality of lower grade surimi by adding protease inhibitor which inhibits the endogenous cysteine proteases (Morrissey et al., 1993).

The cysteine protease inhibitors, cystatins, are subdivided into three subfamilies; stefins, cystatins, and kininogen based on their structural complexity. Stefin (family I) is the smallest inhibitor in the cystatin superfamily, which has a molecular weight of about 11 kDa and lacks of desulfide bonds. Human cystatin A and B (Ritonja et al., 1985) are typical examples. Family II cystatin, which exists in most body fluid and tissues of mammalian and avian, is about 13 kDa with two disulfide bridges. Family II cystatin was purified from chum salmon egg (Yamashita and Konagaya, 1991a). Kininogen (family III) is single chain glycoprotein containing three cystatin-like domains with molecular weight of 68 to 120 kDa (Barret et al., 1986). The related inhibitor originated from botanical seeds such as corn, rice, soybean, and sunflower are more

homologous to the cystatin family I in amino acid sequence, while the lack of 2 intra-disulfide bridges on that inhibitors is similar to the stefin (Abe et al., 1987; Kondo et al., 1990; Kouzuma et al., 1996).

The interaction between proteases and their inhibitor was a target of intensive studying for the last two decades. Protease inhibitors were purified from ovarian fluid carp (Tsai et al., 1996), and egg and muscle of chum salmon (Yamashita and Konagaya, 1991ab), muscle of white croaker (Sangorrin et al., 2001), and Atlantic salmon and Arctic charr (Olenen et al., 2003). Protease inhibitors in muscle of white croaker, hake, Argentine anchovy, castaneta, rough sead, and sea trout were investigated and compared (Borla et al., 1998). The specific inhibitors of cysteine proteases are needed in preventing unwanted destructive proteolysis which can be used in therapy and research (Hernandez and Roush, 2002; Pol and Bjork, 2003), toxic for pest (Gruden et al., 1998; Rogelj et al., 2000), and food industry (Hammann et al., 1990; Pandhere et al., 2000).

There is a strong demand to prevent the deterioration of surimi based product or fish meat by inhibiting digestive fish muscle proteases such as cathepsin and alkaline proteases, etc. The best way to inhibit the fish muscle proteases is to use the natural inhibitor. However, purification of inhibitor from natural resources is very low in recovery, which consequently limits its application potential. Large-scale production of protease inhibitor for food industrial use and biotechnique is highly demanded.

Therefore, the aims of this study were:

1. To purify the protease inhibitor from different fish eggs.
2. To compare the inhibitory activity and investigate the properties of the strongest protease inhibitor.
3. To synthesize DNA encode of its inhibitor and clone into appropriate vector and host.
4. To know protein expression of recombinant inhibitor in *E. coli*.

2. Materials and method

가. Material

Vector pGEX4T-1 (4969 bp), *E. coli* BL 21 (DE3), glutathione-Sepharose 4B, EcoR I, Xho I were purchased from Amersham-Pharmacia Biotech (Uppsala,

Sweden). T4 DNA ligase was obtained from NovagenInc. (Madison, WI, U.S.A.). Polimerase Chain Reaction (PCR) kit, the synthesized DNA, and oligonucleotides were purchased from Bioneer Inc. (Seoul, Korea). Most of other chemicals were purchased from Sigma Chemical (St. Louis, MO, U.S.A.).

Construction recombinant plasmid

The DNA encoded N-terminal amino acid region of glassfish egg protease inhibitor was synthesized Bioneer Inc. (Seoul, Korea). The synthesized DNA sequence was 5'-CGGAATTCCACGCTA ATAGGGTCATGCCTGATATGAACA TGGATTATATGGATGCCCTCGAGCG-3'. The synthesized DNA contained EcoR I and Xho I restriction enzyme site at 5' and 3'end, respectively. PCR was used to amplify the DNA of glassfish egg inhibitor with 30 cycles, which was initiated by 30 s of denaturation at 94 ℃, 30 s of annealing at 60 ℃, and extension at 72 ℃ and then final extension step was at 72 ℃ for 2 min in a DNA thermal cycles (Eppendorof personal PCR system). The forward primer was 5'-CGGAATTCCACGCTAATAG-3' and the reverse primer was 5'-CGGCTCGAGGGCATCCAT-3'. After PCR reaction, PCR product and pGEX4T-1 plasmid were restricted by EcoR I and Xho I at 37 ℃ overnight and ligated at 16 ℃ for 2 hr. Ligated plasmid was transformed to *E. coli* XL-1-Blue by Inoue method (Sambrook and Russell, 2001). *E. coli* transformant was screened by antibiotic (50 g/mL ampicillin) selection. Plasmid DNA was isolated from positive colonies and sequenced to check inserted DNA. The recombinant plasmid with correct in-frame coding sequence of inserted DNA was transformed into the host *E. coli* strain BL21 (DE3) for protein expression.

나. Expression of fusion protein

Positive colonies of *E. coli* strain BL21 in 30 mL LB broth containing 50 g/mL ampicillin were used for protein expression. In brief, each bacterial colony was grown in 30 mL LB broth containing 50 g/mL ampicillin until the optical density at 590 nm reached 0.6. Isopropyl-D-thiogalactopyranoside (IPTG) (Bio Basic Inc., Canada) was then added to the final concentration of 1 mM. The cell was harvested by centrifugation of 1 mL culture at 1, 2, 3, 4, and 6 hr after inducing with IPTG. Before inducing, 1 mL culture was also harvested as 0 hr. The cell pellet was boiled in 50 L of loading buffer for 10 min and electrophoresed on 12.5% polyacrylamide gel as described by Laemmli (1970).

다. Purification of recombinant protein

Recombinant fusion proteins were obtained from 250 mL cultures. The overnight culture of transformed *E. coli* (5 mL) was inoculated into 250 mL of fresh LB-Amp broth. The culture was incubated at 27 ℃ for 4 hr with vigorous shaking. When optical density of culture at 590 nm was reached 0.6, IPTG was added to final concentration of 1 mM in culture. Culture was further incubated for 5 h. The cells were harvested by centrifugation at 9000×g for 10 min at 4 ℃ and incubated -20 ℃ for 2 hr prior lysis. One gram of frozen cells was thawed, resuspended in 10 mL of phosphate buffer saline (PBS), and disrupted by sonication. Triton X-100 (20%) was added to a final concentration of 1%, mixed gently for 30 min, and then lysate was centrifuged at 15,000×g for 20 min at 4 ℃. 8 mL supernatant was loaded into 1 mL GSTrap FF column (Amesham Biosciences Ltd., Uppsala, Sweden), equilibrated by PBS (pH 7.2) and eluted by elution buffer (50 mM Tris-HCl, pH 8.0 with 10 mM reduced glutathione). The purified fusion protein was assessed by SDS-PAGE stained by commassie brilliant blue. The fusion protein was finally dialysed against 10 mM Tris-HCl, pH 8.0 and digested with 100 U of thrombin at 37 ℃ for 6 hr. After cleavage with thrombin, the recombinant inhibitor was purified by Sephacryl HR 100 (Amersham Biosciences Ltd., Uppsala, Sweden) column (2.6 x 100 cm), packed and equilibrated with 50 mM ammonium bicarbonate buffer, pH 7. The protein peak was assayed inhibitory activity against papain.

3. Result and disccussion

가. Plasmid expresion

Synthesized DNA of N-terminal region glassfish egg protease inhibitor was successfully amplified by the standard PCR protocol. Agarose gel electrophoresis pattern of PCR product before and after digestion with EcoR I and XhoI is shown in Fig. 32. Molecular weight of synthesized DNA was 65 bp. The agarose gel electrophoresis pattern of plasmid pGEX4T-1 before and after digestion with EcoR I and Xho I as a vector is shown in Fig. 33. Mgration of circle formof pGEX4T-1 in gel was faster than in linear form, ~ 4900 bp, after digestion.

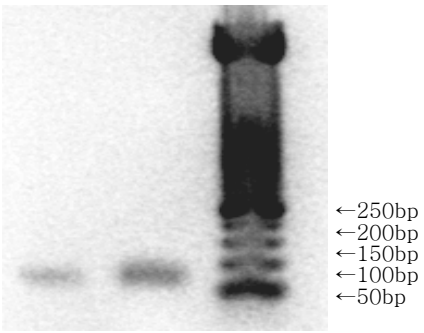

Fig. 1. Agarose gel electrophoresis pattern of PCR product of synthesized DNA of N-terminal part of glassfish protease inhibitor. A, after digestion with at EcoR I and Xho I restriction site; B, before digestion; M, DNA size marker (50 bp Ladder).

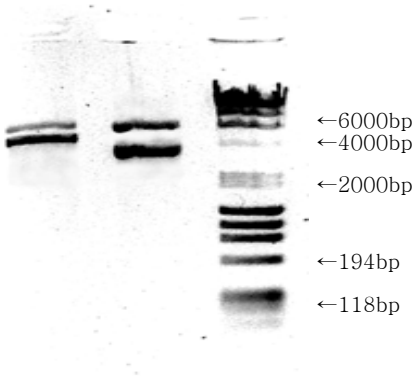

Fig. 2. Agarose gel electrophoresis pattern of pGEX4T-1 plasmid digestion with EcoR I and Xho I restriction enzyme. A, after digestion; B, before digestion; M, DNA size marker (mixture of DNA-Hind III digest and DNA X174 Hinc II digest)

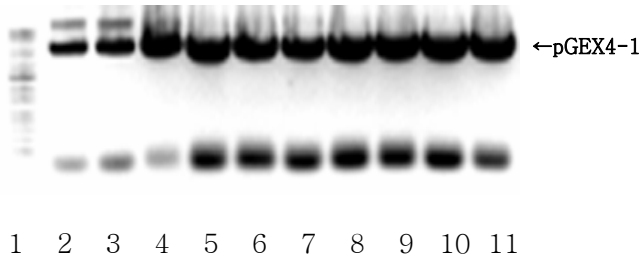

Fig. 3. Agarose gel electrophoresis pattern of miniprep of several colonies *E. coli* XL-1-Blue in Luria Bertani (LB) agar containing 50 g/mL ampicillin. Lane 1, DNA size marker; Lanes 2-11, some positive colonies of E. coli XL-1-Blue contain pGEX4T-1 plasmid.

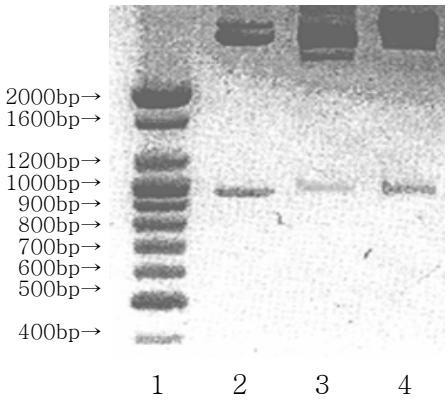

Fig. 4. Agarose gel electrophoresis pattern of pGEX4T-1 containing insert DNA cut at EcoR I and Pst I restriction sites. Lane 1, DNA marker (Bioneer D-1030); Lane 2, pGEX4T-1 without insert DNA; Lane 3 & 4, pGEX4T-1 containing insert DNA.

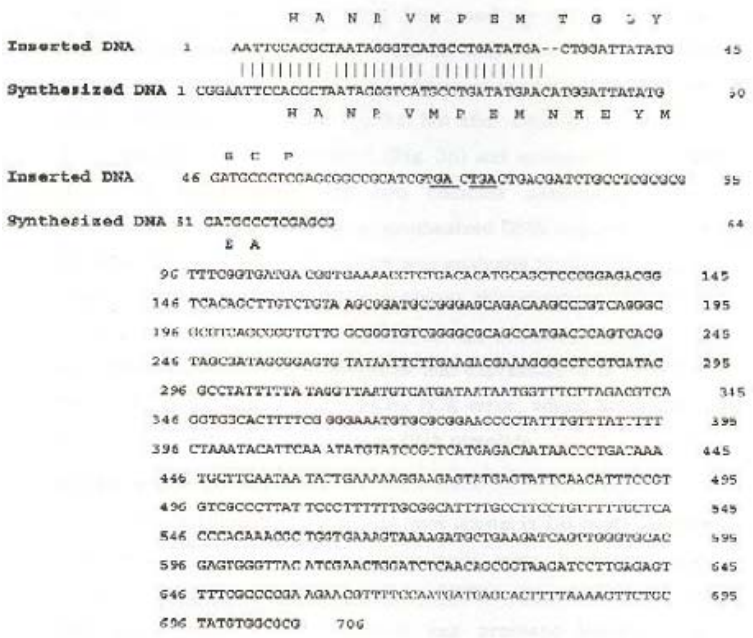

Fig. 5. Sequence of inserted DNA in a part of pGEX4T-1 from EcoR I to Pst I restriction site and its homology with synthesized DNA (analysis with EMBOSS-Align). Codon with bold and underline are stop codons.

After ligation of synthesized DNA with pGEX4-1, transformation into *E. coli* XL-1 Blue, and spreading on Luria-Bertani (LB) agar containing 50 g/mL ampicillin, some colonies grew. Agarose gel electrophoresis pattern of the isolated DNA from several colonies is shown in Fig. 3. But after cutting at EcoR I and Pst I restriction site of pGEX4-1 (Fig. 4) and sequencing that part (Fig. 5), there were just two colonies containing correct recombinant vector. Similarity of synthesized DNA sequence before and after inserted was 78%, which was analyzed by EMBOSS-Align (www.ebi.ac.uk/emboss/align). It indicates that synthesized DNA of N-terminal region of low MW glassfish egg protease inhibitor was not cloned well by pGEX4T-1 vector and expressed in E. coli XL-1 Blue system. It might be caused by PCR error, which produced of PCR product with 78% similarity to DNA tamplate.

나. Protein expression

Recombinant *E. coli* BL-21 was grown in LB broth medium containing 50 g/mL and then the cells were harvested after Isopropyl--D-thiogalactopyranoside

(IPTG) induction for 6 hr. The fusion protein GST-glassfish egg protease inhibitorwas expressed in soluble form by IPTG induction (Fig. 6). SDS-PAGE analysis showed the presence of a predominant induction band with molecular weight around 29 kDa.

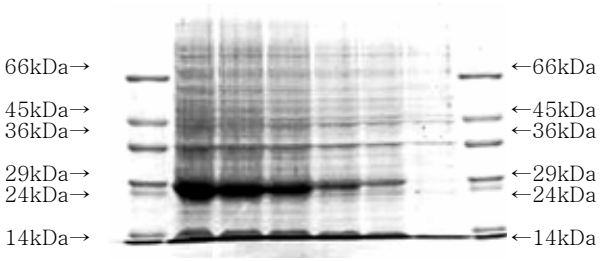

Fig. 6. SDS-PAGE pattern of fusion Glutathion-S-transferase (GST)-recombinantprotein expression with E. coli B21 system. Lane 1 & 8, protein marker (Sigma MW-SDS-70L); Lane 2-6, after induction for 6, 4, 3, 2, and 1 hr.; Lane 7, 0 hr after induction

GSTrap FF affinity chromatography pattern of fusion protein is shown in Figure 7. Small peak on SDS-PAGE analysis showed one protein band as purified GST- recombinant glassfish protease inhibitor with MW 29 kDa (Fig. 8).

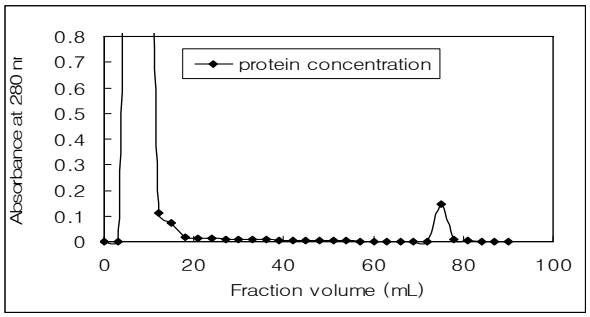

Fig. 7. GSTrap FF affinity chromatography pattern of GST-glassfish egg protease recombinant inhibitor.

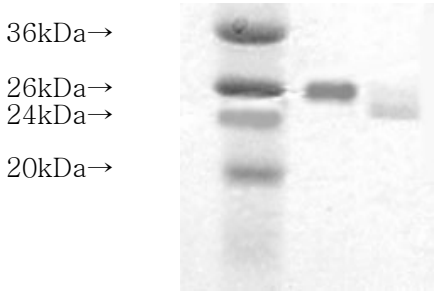

Fig. 8. SDS-PAGE pattern of fusion GST-recombinant protein after and before cut with thrombin. Lane 1, Molecular size marker; Lane 2, GST-Recombinant protein; Lane 3, GS

Purified GST- inhibitor recombinant glassfish was cleaved by thrombin. SDS-PAGE analysis showed one band as GST with MW 26 kDa, while protein band of recombinant protease inhibitor was not shown. After digestion, GST and recombinant protease inhibitor was separated by Sephacryl 100 HR chromatography (Fig. 9). Specific inhibitory activity against of Peak II as a recombinant purified inhibitor was 7.117 U/mg (Table 14), while Peak I (GST) showed no inhibitory activity.

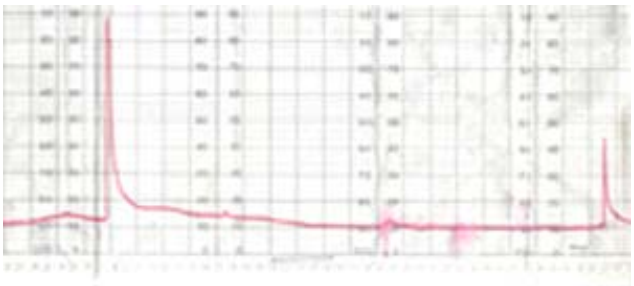

Fig. 9. Sephacryl HR-100 chromatography of fusion GST (Peak I) and recombinant protein (Peak II).

The fusion protein, Glutathion-S-Transferase (GST), was a good system for expressing soluble form of protein in E. coli and secreted into the cytoplasmic space of the host cell (Davis et al., 1999). Recombinant plasmid pGEX-4T-1 containing inserted DNA of N-terminal region of glassfish egg protease inhibitor was transformed to E. coli BL21 (DE3) cells. The BL21 genotype F-, ompT, hsdS (rB-,mB-) carries a chromosomal copy of the gene for T7 RNA polymerase under control of LacUV5 promoter (Studier and Moffat, 1986). In addition, the pGEX-4T-1 expression vector with a tac promoter for chemical

induced using IPTG and an internal lac I q gene for use in any *E. coli* host is considered to be a powerful expression vector. After IPTG induction, the recombinant GST-glassfish egg protease inhibitor was over-expressed in the *E. coli* BL21 transformant.

After purification of recombinant fusion GST-glassfish egg protease with GSTrap FF affinity chromatography, further cleaved of the fusion protein by thrombin and then separated by Sephacryl HR-100 gel permeation chromatography, 0.0125 mg of recombinant protease inhibitor was produced from 250 mL culture. One mg of recombinant peptide of several rat cystatin S was produced from 1 L culture, which was also expressed with pGEX-4T-2 vector and *E. coli* BL-21 (Bedi et al., 1998). Low production of recombinant glassfish egg protease might be caused by small density harvested cell. Dissolved oxygen and pH in the culture was not controlled, therefore culture of recombinant *E. coli* BL-21 might be not in optimal condition to produce maximum cell mass.

Table 3. Inhibitory activity of recombinant protease inhibitor against papain

Inhibitor	Total protein (mg)	Total activity (Unit)	Specific activity (Unit/mg)
GST-recombinant	3.573	0.10	0.033
Recombinant	0.0125	0.885	7.117
Natural	0.15	2.87	19.70

GST : Glutathione S-transferase (fusion protein)

Specific inhibitory activity of recombinant protease inhibitor, 7.117 U/mg, was lower than 19.70 U/mg specific inhibitory activity of natural protease inhibitor from glassfish egg. Because 7 deduced amino acids sequence of recombinant protease inhibitor was changed, especially Met and Asn residues, it might cause the decrease in inhibitory activity. Bode et al. (1988) said that Met residue is one of the important residues in the active site of protease inhibitor.

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제 7 절 재조합 효모의 연어알 단백분해효소저해제의 특성

1. The stability of plasmid used in the recombinant yeast

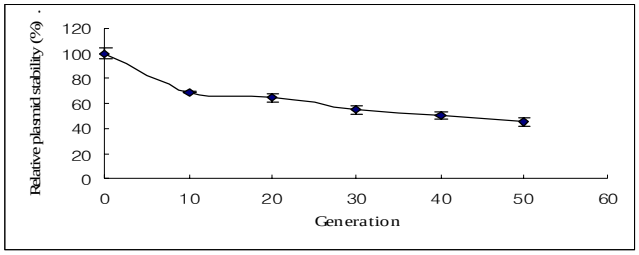

Fig. 1. Plasmid stability of Recombinant yeast

The pYES2/NT carried 2u origin for episomal maintenance and high copy replication of the plasmid in yeast, generally 10-40 copies per cell was guaranteed, the plasmid stability was maintained close to 50% till 50th generation.

2. Optimization of overexpression

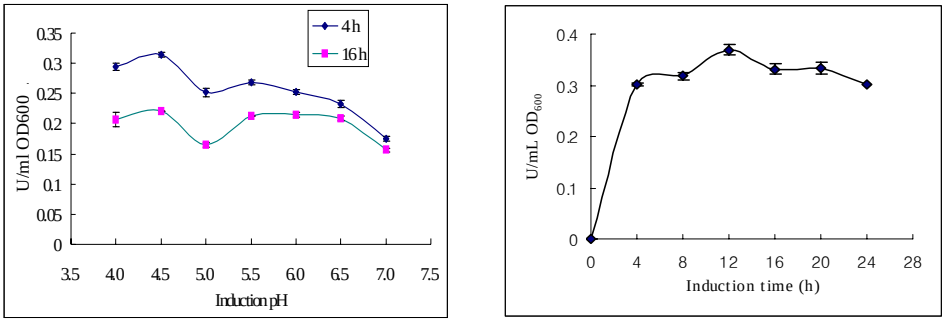

Fig. 2. Optimization of induction pH and duration for recombinant cystatin

The induction pH (Fig. 2) effected greatly on the yield of recombinant cystatin, and at pH 4.5 the highest expression was obtained. Further increase of pH resulted in another peak of yield at pH 5.5, which was lower than that at pH 4.5. With the increase of induction time from 4 h to 16 h, the yield difference between pH 4.5 and 5.5 decreased. Considering further application of recombinant cystatin into surimi gel, pH 5.5, close to neutral, was chosen as the optima induction pH 12 h was chosen as the optima induction time.

3. Purification and characterization of recombinant cystatin

Table 1. Summary of the purification of recombinant cystatin from YPH 499 transformant

Steps	Total protein (mg)	Total activity[b] (U)	Specific activity (U/mg)	Yield (%)	Purity (fold)
Cell lysate[a]	30	40	1.33	100	1
His-affinity	2.75	24.49	7.45	61.23	5.60

a: The starting volume was 1000 mL cultivated broth.
b: The inhibitory activity was measured as decrease in papain activity. One unit of inhibitory activity was defined as the changes in absorbance at 440 nm per 30 min.

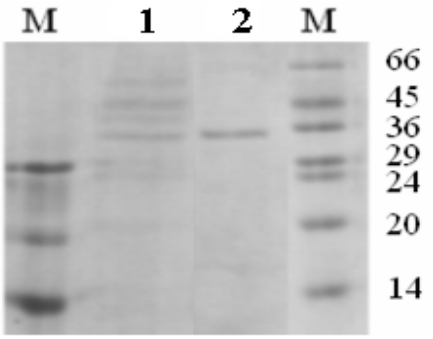

Fig.3. Purification of recombinant inhibitor. 1, cell lysate; 2, purified recombinant cystaitn.

The recombinant cystatin was formed as a fusion protein, which was convenient for both detection and purification. After purification, the specific inhibitory against papain reached 7.45 (Table 1), which was higher than the inhibitor purified from chum salmon egg by K. Y Kim (2006).

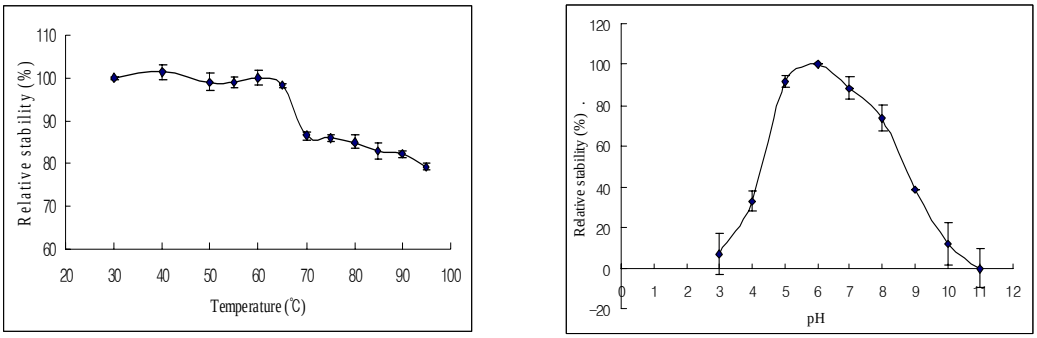

Fig.4. Thermal and pH stability of recombinant cystatin

The recombinant cystatin was very stable at temperature lower than 65 ℃. Further higher temperature after 70 ℃ resulted in gradually decrease of stability. The recombinant cystatin was relatively thermal stable. The recombinant cystatin was relatively unstable at acidic condition. After the pH increased to 5.0, the inhibitory activity was greatly recovered to 91.86%, and at pH 6.0 reached 100%. As the pH increased to neutral and then alkaline scope, the inhibitory activity decreased gradually.

제 8 절 천연 효소저해제가 함유된 수산식품의 품질향상 연구

1. Introduction

Cystatin is the group name of cysteine protease inhibitors, mainly consisting of four types of molecular forms; families 1, 2, 3, and 4. Cystatins are potent, non-covalent, and competitive inhibitors of cysteine proteases, such as papain, ficin, and cathepsins, which should regulate the activity of endogenous cysteine proteases that mediate proteolysis and tissue damage. At beginning the attention was focused on mammalian and plant cystatins due to their wide distribution among tissues and body fluids. Since Yamashita and Konagaya had isolated two different cysteine protease inhibitors from chum salmon egg, a lot of studies have been focused on fish cystatins. Thereafter, cystatins were purified from ovarian fluid of carp, egg of Alaska pollock and glassfish, egg and skin of chum salmon, liver of rainbow trout, and Chinese sturgeon. Fish cystatin is considered to be involved in pathological processes. Its potential values in the fish disease prevention and cure as well as seafood process industry have drawn wide attention.

Surimi, a stabilized fish myofibrillar protein, is the primary ingredient in surimi based products. Gel functionality, such as texture and color, of surimi seafood is the most important aspect of product quality. However, autolysis by endogenous heat stable proteases causes an irreversible destruction of the surimi gel structure, especially at temperatures close to 60 ℃. Among the numerous proteases present in fish muscle, cysteine endoproteases have the most serious effects on texture because of their thermo stability and ability to cleave internal peptide bonds. Calpains, Ca^{2+}-dependent cysteine proteases including μ-calpain and m-calpain (the low and high calcium requiring calpains, respectively) were also found to cause texture deterioration in some fish. The proteases causing autolysis in Alaska pollock surimi are mainly cysteine proteases together with serine proteases. Thus, cystatins have been considered to be applied to inhibit proteases in surimi to prevent the gel weakening. However, the low recovery and long time of purification by the common methods limited the availability of cystatins and their application in surimi. Therefore, a large amount production of

cystatin by recombinant technology could be an alternative for the application of cystatin. Human and chicken cystatins were overexpressed either by *Escherichia coli* or *Pichia pastoris* and the recombinant chicken cystatin could inhibit the gel weakening of mackerel surimi.

Chum salmon cystatin was equipotent to chicken egg white cystatin in the papain inhibitory assay. It was a homolog of mammalian cystatin C. However, no further characterization and application of chum salmon cystatin were investigated. Saccharomyces cerevisiae is usually used as a host for heterologous production historically, and also it is regarded as GRAS (generally recognized as safe) organism. Because an overwhelming wealth of information on genetics, molecular biology, and physiology has been accumulated, this traditional species is the best characterized eukaryotic system today. In this study, the chum salmon cystatin was overexpressed in *Saccharomyces cerevisiae*, and the recombinant chum salmon cystatin (RC) was purified and characterized. The effect of RC on preventing the gel weakening of Alaska pollock surimi was investigated.

2. Materials and Methods

가. Yeast strain, plasmid, media, and raw materials

Saccharomyces cerevisiae YPH 499 incorporating the pYES2/NT_C (GAL promoter, uracil marker) vector was purchased from Invitrogen (San Diego, Calif., U.S.A.). Synthetic minimal defined medium (SD medium) minus uracil [0.67% yeast nitrogen base (YNB), 2% dextrose, 0.01% (adenine,arginine, cysteine, leucine, lysine, threonine, and tryptophan), 0.005% (aspartic acid, histidine, isoleucine, methionine, phenylalanine, proline, serine, tyrosine, and valine)] were used for the culture of *Saccharomyces cerevisiae* YPH 499. KA grade frozen Alaska pollock (*Theragra chalcogramma*) surimi was obtained from Golden Alaska Seafood Co. (Seattle, WA, U.S.A.). Dried egg white (EW) was obtained from Inovatech Inc. (Abbotsford, B.C., Canada). Papain (from papaya latex, 18 U/mg), cathepsin L (from human liver, 4 U/mg), azocasein, and N-CBZ-phenylalanyl-arginine-7-amido-4-methylcoumarin (Z-Phe-Arg-NMec) were purchased from Sigma (St. Louis, Mo., U.S.A.). Other chemicals used in this study were of the first grade.

나. Heterologous expression

The open reading frames encoding for chum salmon cystatin were synthesized according to Yamashita and Konagaya and amplified by PCR using the cystatin-5BH and cystatin-3E primers, which were 30-mer 5′ cgggatccatgatcatggaatggaagatcg 3′ and 31-mer 5′ ggaattcttaactttcacactggttcttgac 3′, respectively. The PCRs for the plasmodial constructs were performed using Pfu polymerase (Invitrogen, San Diego, Calif., U.S.A.). The cycling profile included 3 min at 95 ℃for 1 cycle and 35 cycles of 1 min at 95 ℃ 1.5 min at 42 ℃ and 3 min at 60 ℃ The generated PCR products were digested with BamH I and EcoR I, and ligated with vector pYES2/NT_C previously digested with the same enzymes. Thereafter, the recombinant plasmid pYES2/NT_C (cystatin) was verified by sequencing and transformed into *Saccharomyces cerevisiae* YPH 499 strain using the lithium chloride method. Transformants were selected and maintained on SD medium minus uracil. Preliminary experiments were carried out to determine the optimal pH and time in glucose-free galactose-containing SD medium minus uracil for the induction of recombinant protein. Total protein was extracted from cells after the breakage of the cells using glass beads in the buffer (50 mM sodium phosphate containing 50 mM NaCl, pH 8.0). Protein extract was passed through a membrane filter (0.22 μm 47 mm, Millipore, U.S.A.) and the cystatin in the filtrate was purified by His-select nickel affinity chromatography according to the manufacturer's instruction (Sigma, St. Louis, Mo., U.S.A.). The active fractions which showed inhibitory activity against papain in the eluate were pooled and dialyzed against acetate buffer (25 mM sodium acetate containing 1 mM EDTA, 1 mM β-mercaptoethanol, and 50 mM NaCl, pH 6.0) before lyophilization.

다. Inhibitory activity of RC against papain and cathepsin L

Inhibitory activity of RC against papain was determined using azocasein as a substrate. 200 μL of RC (0.05 mg/mL in acetate buffer) was added to 100 μL of papain solution (0.25 mg/mL in acetate buffer). The RC-papain mixture was incubated at 37 ℃or 5 min and then added with 250 μL of azocasein solution (3.2 mg/mL in acetate buffer) pre-incubated at 37 ℃ This mixture was incubated at 37 ℃for 30 min and then 700 μL of 20% trichloroacetic acid (TCA) was added to stop the reaction. The control was prepared by substituting 200 μ

L of inhibitor solution with 200 μL of acetate buffer. Blank was prepared by adding 700 μL of 20% TCA in advance before adding 250 μL of substrate solution. 720 μL of supernatant obtained after the centrifugation at 10000 ×g for 5 min was added to 800 μL of 1 N NaOH for developing color. Papain activity was expressed as the absorbance at 440 nm. One unit of protease activity was defined as the amount of enzyme that induced an increase of 1.0 in absorbance at 440 nm after incubation at 37 ℃ for 30 min. Inhibitory activity was calculated by subtracting papain activity without inhibitor from that with inhibitor. One unit of inhibitory activity was defined as one unit decrease of papain activity.

Inhibitory activity of RC against cathepsin L was determined using Z-Phe-Arg-NMec as a substrate. Three ng of cathepsin L in 500 μL of diluent of 0.1% Brij 35 in water was added with 250 μL of assay buffer (340 mM sodium acetate containing 60 mM acetic acid and 4 mM EDTA, pH 5.5) or RC in assay buffer. One min was allowed for the activation of cathepsin L and temperature equilibration in a bath at 30 ℃ and 250 μL of the substrate solution (1 mM Z-Phe-Arg-NMec in dimethyl sulfoxide) was then added. After standing for exactly 10 min, 1 mL of stopping solution (100 mM sodium monochloroacetate, 30 mM sodium acetate, and 70 mM acetic acid, pH 4.3) was added. One unit of protease activity was defined as the amount of enzyme required to produce 1μmol AMC per min at 30 ℃ Inhibitory activity was calculated by subtracting protease activity without inhibitor from that with inhibitor. One unit of inhibitory activity was defined as one unit decrease of cathepsin L activity.

라. pH and thermal stability of RC

pH and thermal stability of RC was assayed according to Tzeng and others (2002). RC in various buffers, pH 3.0 to 5.0 (50 mM sodium citrate buffer), pH 6.0 to 8.0 (50 mM sodium phosphate buffer), pH 9.0 to 10.0 (50 mM Clark and Lubs solutions), and pH 11.0 (50 mM disodium phosphate and sodium hydroxide solution) was incubated at 37 ℃ After 30 min incubation, an equal volume of 0.2 M acetate buffer (pH 6.0) was added, and the residual inhibitory activity against papain was determined as described above. RC in acetate buffer was incubated at temperatures ranging from 30 to 95 ℃for 30 min. After being

cooled in icy water for 30 min, the residual inhibitory activity against papain was determined as described above.

마. Inhibitory activity of RC against surimi autolysis

The inhibitory activity of RC against surimi autolysis was measured according to the method of Morrissey and others (1993). Surimi (3g) was prepared with RC to a final concentration of 10, 30, 60, 100, 150, and 200 μg/g. The mixture was then immediately incubated in a water bath at 55 ℃ for 1 h. Autolysis was stopped by the addition of 27 ml of 5% cold TCA solution. This was homogenized for 2 min and kept on ice for 1 h, and then centrifuged at 8000 × g for 5min. TCA-soluble peptides in the supernatant were analyzed according to Lowry method by Bio-Rad kit. Inhibition of autolysis was calculated using the following equation:

Autolysis inhibition (%) = 100 × [(TC - TCb) - (TI - TIb)]/(TC - TCb),

where, TC is TCA-soluble peptides of control (without RC) incubated at 55 ℃ TCb is TCA-soluble peptides of control incubated at 0 ℃ TI is TCA-soluble peptides of sample (with RC) incubated at 55 ℃ TIb is TCA-soluble peptides of sample incubated at 0 ℃

바. Surimi gel preparation

Frozen surimi (150 g) was partially thawed at 4 ℃for 2 - 3 h, cut into small pieces, and chopped in a mixer for 4 min with 2% (w/w) NaCl. RC was added to a final concentration of 10, 30, 60, 100, 150 μg/g, respectively. Water content was adjusted to 78%. Chopping was conducted for 5 min at temperature maintained below 10 ℃ The paste was stuffed into polyvinylidine casing with a diameter of 3.0 cm and both ends were sealed tightly. The paste was incubated at 55 ℃ for 1 h, followed by heating at 90 ℃ for 20 min in a water bath. This sample was referred to as "modori gel". A directly cooked gel was heated at 90 ℃for 20 min and referred to as "directly heated gel". Surimi with the addition of EW at 20000 μg/g was incubated and heated as did modori gel. After heating, all gels were immediately cooled in iced water for 30 min and stored at 4 ℃ overnight prior to analysis.

사. Texture analysis

Texture analysis of surimi gels was carried out using a Compac-100 Rheometer (Sun Scientific Co., Ltd., Tokyo, Japan). Gels were equilibrated at room temperature for 2 h and cut into pieces of 20 mm in length before analysis. Breaking force (strength) and deformation (cohesiveness/elasticity) were measured by the Rheometer equipped with a round probe (20 mm in diameter, 30 mm/min depression speed).

아. Whiteness measurement

Samples (30 mm in diameter × 25 mm in length) from each treatment were subjected to whiteness measurement using a JP7100F colorimeter (Juki Corp., Tokyo, Japan) at ambient temperature. CIE L*, a*, and b* values were measured. Whiteness was calculated using the following equation:

Whiteness = L*−3b*

자. Determination of Expressible drip

Expressible drip was measured according to the method of Ng (1987). Cylindrical gel samples were cut to a thickness of 5 mm, weighed (X), and placed between two pieces of Whatman paper No.1. A standard weight (5 kg) was placed on the top of the sample for 2 min, and then the sample was removed from the papers and weighed again (Y). Expressible drip was calculated with the following equation and expressed as the percentage of sample weight:

Expressible drip (%) =100 ×[(X−Y)/X]

차. Protein pattern of surimi gels

SDS-PAGE was performed according to the method of Laemmli (1970). 18 mL of 5% (w/v) SDS solution was added to 2 g surimi gel. The mixture was homogenized using an IKA homogenizer (Selangor, Malaysia) at a speed of 11000 rpm for 1 min and the homogenates were incubated at 85 ℃ in a water bath for 1 h to dissolve all proteins. The samples were centrifuged at 10000 × g for 5 min to remove undissolved debris. Solubilized samples were mixed at 1:1 (v/v) ratio with the sample buffer (500 mM Tris - HCl containing 2.5% SDS, 0.01% bromophenol blue, 20% glycerol, and 10% -mercaptoethanol, pH 6.8) to give a final protein concentration of 4 mg/mL. After incubated at 95℃for 3 min,

5 μL aliquots from each prepared samples were loaded into the polyacrylamide gel consisted of 10% separating gel and 4% stacking gel, and subjected to electrophoresis. The proteins were separated on a Mini-Protein II unit (Bio-Rad Laboratories, Hercules, Calif., U.S.A.) at a constant voltage of 150 V for 60 min. After separation, the proteins were stained with 0.02% (w/v) Coomassie brilliant blue R-250 (Bio-Rad Laboratories) in methanol - acetic acid - water solution (30:10:60, v/v/v) and destained with 7% (v/v) acetic acid.

카. Statistical analysis

Data were subjected to analysis of variance (ANOVA). Comparison of means was carried out by Duncan's multiple-range test. Analysis was performed using a SPSS package (SPSS 12.0 for windows, SPSS Inc., Chicago, IL, U.S.A.).

3. Results and Discussion

가. Inhibitory activity of RC against papain and cathepsin L

Table 1. Comparison of inhibitory activity of recombinant chum salmon cystatin with others against papain and cathepsin L

Inhibitor	Specific inhibitory activity (U/mg)	
	Papain	Cathepsin L
Glassfish egg inhibitor[a]	19.7	36.84
Chicken egg white cystatin[a]	37.71	16.05
Chum salmon egg kininogen[b]	4.67	28.02
RC	7.45	10.24

a Ustadi and others 2005b; b Kim and others 2006; Means obtained from four replications.
*RC, recombinant chum salmon cystatin.

The inhibitory activity of RC against papain and cathepsin L is shown in Table 1. The inhibitory activity of RC was compared with the activities of other inhibitors such as glassfish egg inhibitor (Ustadi 2005), chicken egg white cystatin, and chum salmon egg kininogen (Kim 2006) which were analyzed using the same method. Chum salmon cystatin was reported to be equipotent to chicken egg white cystatin against papain. However, RC showed lower inhibitory activities against both papain and cathepsin L than those of egg white cystatin. And glassfish egg inhibitor also showed higher inhibitory activity than that of RC. Decrease in the specific inhibitory activity of RC was supposed to be due

to the recombinant technology, in which RC was expressed as a fusion protein. In order to simplify the purification of RC, pYES2/NT_C was used as an expressing vector for chum salmon cystatin. Therefore, a fusion protein part was added to RC after expression and purification. Thus, his-select nickel affinity chromatography could be used for purification of RC and the inhibitory activity of RC might decrease to some level. Although the specific inhibitory activity of RC decreased a little, RC still showed higher inhibitory activity against papain than did chum salmon egg kininogen.

나. pH and thermal stability of RC

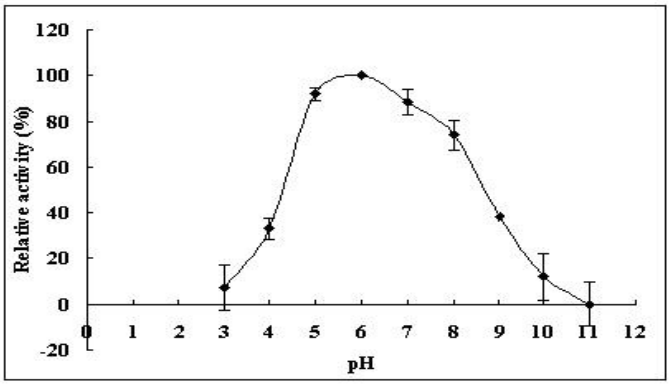

Fig.1. pH stability of recombinant chum salmon cystatin.

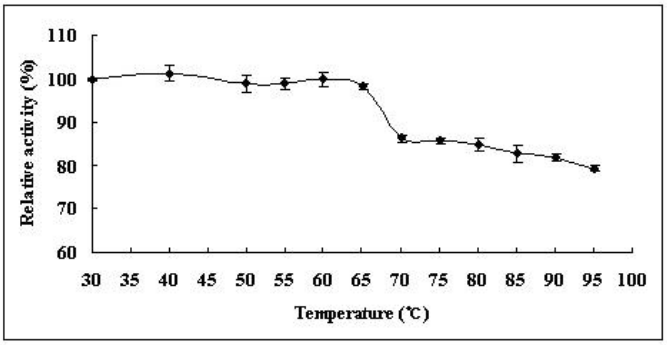

Fig.2. Thermal stability of recombinant chum salmon cystatin.

The pH and thermal stabilities of RC are shown in Figure 1 and Figure 2, respectively. RC was relatively unstable at acidic condition; only 32.98% activity was remained at pH 4.0. When the pH was adjusted to 5.0, the inhibitory activity was greatly recovered to 91.86% and reached 100% at pH 6.0. As the pH increased to neutral and then alkaline conditions, the inhibitory activity

decreased gradually, which was consistent with the result of two cysteine protease inhibitors from chum salmon egg. Based on the cysteine proteases mainly active in the weak acidic range, the pH stability of RC could assure the effective inhibition of corresponding proteases, which favors its application in surimi industry.

As shown in Figure 2, RC was stable at temperature lower than 65 ℃ Because the autolysis of surimi gel was mainly occurred at temperature around 50 - 60 ℃ RC should be capable of preventing the gel weakening of surimi due to its heat tolerance. As the temperature increased, the inhibitory activity of RC decreased significantly at 70 ℃ Further increase of temperature resulted in gradual decrease in RC stability. The decrease of stability was due to partly unfolding of RC. Since the inhibitory activity was kept about 80% at 95 ℃ RC was relatively thermo stable in which the fusion protein part was considered to enhance the thermo stability of RC(Wrenger 2005). Therefore, both the pH and thermal stabilities enlightened the application of RC in surimi processing to prevent gel weakening.

다. Inhibitory activity of RC against surimi autolysis

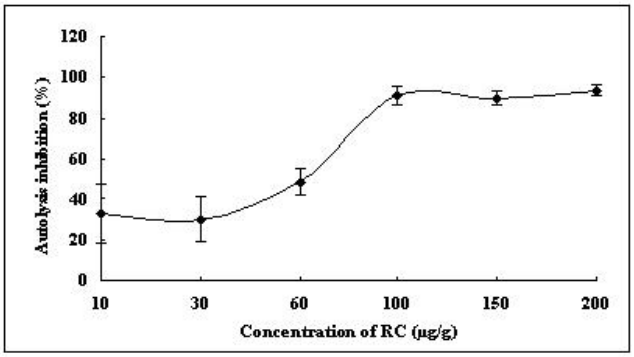

Fig. 3. Inhibitory activity of recombinant chum salmon cystatin against surimi autolysis. RC, recombinant chum salmon cystatin.

Marked inhibition of surimi autolysis was observed when RC was added (Fig.3). The more amount of RC was added to surimi, the higher was the inhibition of autolysis with less than 30% at 10 μg/g to 90% at 100 μg/g. The further increase in RC addition showed no significant increase in the inhibition of autolysis ($P < 0.05$). The proteases causing autolysis in Alaska pollock surimi

are cysteine proteases such as cathepsin B, S, L, and others, and serine proteases. Because cathepsin L is still active and bound to myofibril after minced and washed, the inhibition of cathepsin L could greatly affect the autolysis in surimi gel. The inhibition of cathepsin L was determined not only by the specific activity of inhibitor, but also by the effective proximity of the applied inhibitor. Hence, the cysteine protease inhibitor with high specific inhibitory activity and relatively low molecule weight has a great advantage of application in surimi processing. Therefore, chum salmon cystatin was overexpressed for a large production, and RC at 100 μg/g had the highest inhibitory activity against surimi autolysis.

라. Effect of RC on textural properties of surimi gel

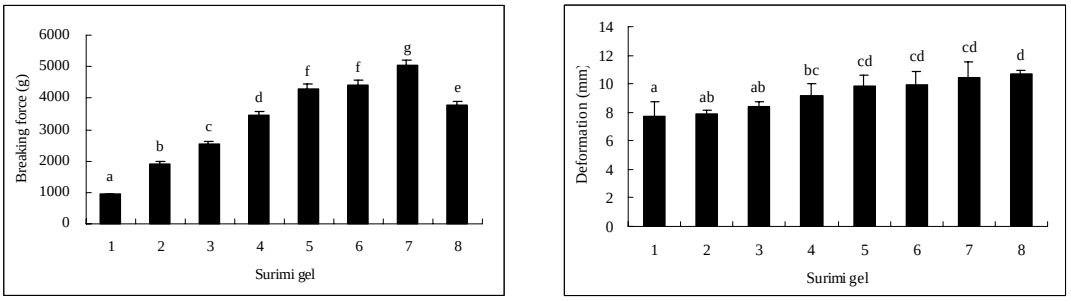

Fig. 4. Breaking force and deformation of surimi gels added with different concentrations of recombinant cystatin. 1: surimi gel without inhibitors; 2–6: surimi gel added with 10, 30, 60, 100, 150 g/g recombinant cystatin 7: surimi gel added with 60 g/g chymostatin, 8: surimi gel added with 2% egg white powder. Bars represent the standard deviation from five determinations. Different letters in the same grade surimi indicate significant differences ($p < 0.05$).

Formation of large aggregates is presumably a prerequisite for the formation of a good elastic gel. Elevated temperature during heating resulted in more oxidation of sulphydryl groups with a subsequent disulfide bond formation. Endogenous proteases, especially heat activated and heat stable protease, have been known to play a detrimental role in surimi gel quality. Thus, the breaking force (Figure 4a) and deformation (Figure 4b) of modori gel increased because

the addition of RC inhibited the gel autolysis. The breaking force of modori gel without RC was near 1000 g. When RC was added, breaking force of modori gel significantly increased ($P< 0.05$). The addition of RC at 100 μg/g resulted in the maximal breaking force of modori gel ($P< 0.05$), which was about 4.5 times higher than that of modori gel without RC, and nearly 2 times higher than that of the directly heated gel. Further increase in RC addition showed no more increase in breaking force ($P < 0.05$). The breaking force of modori gel with RC at 100 μg/g was about 14% higher than at 20000 μg/g (2%, w/v) of EW. EW is applied to inhibit autolysis in surimi gel, and it's also nutrition additional and whitening helpful. Besides cysteine and serine protease inhibitors such as egg white cystatin and antitrypsin, EW itselfwas found to have gel forming ability. Thus, addition of EW could greatly enhance the gel texture. However, because of off odor produced during heating in surimi processing and the outbreak of avian influenza (AI), EW has been trying out to be replaced with another alternative. The changes in the deformation of modori gels were similar to that in breaking force. The addition of RC at 100 and 150 μg/g had the highest deformation ($P< 0.05$), which was about 30% and 8% higher than those of modori gel without RC and directly heated gel, respectively. No significant difference in the deformation of modori gel was determined between the additions of RC at 100 μg/g and EW at 20000 μg/g ($P < 0.05$).

마. Effect of RC on whiteness and expressible drip of modori gels

Table 2. Whiteness and expressible drip of modori gels added with recombinant chum salmon cystatin at different concentrations

Sample	Whiteness	Expressible drip (%)
Directly heated gel	51.05 ±0.98^{abc}	6.54 ± 0.49^{a}
Control	49.78 ± 1.25^{a}	17.85 ± 3.29^{d}
10 μg/g RC	49.73 ± 1.14^{a}	13.82 ± 1.14^{c}
30 μg/g RC	50.50 ± 0.63^{ab}	11.14 ± 0.76^{b}
60 μg/g RC	51.84 ± 0.72^{bcd}	9.56 ± 0.30^{b}
100 μg/g RC	52.60 ± 1.08^{cd}	6.27 ± 0.32^{a}
150 μg/g RC	52.61 ± 1.05^{cd}	5.78 ± 0.22^{a}
20000 μg/g EW	52.58 ± 0.96^{cd}	5.07 ± 0.17^{a}

a, b, c, d Means in the same column with different superscripts are significantly different ($P < 0.05$); Values are given as mean ± SD from triplicate determinations.
* RC, recombinant chum salmon cystatin; EW, egg white.

The texture and whiteness of surimi gel were main quality parameters determining the market value of surimi-based products. Generally the additives such as bovine plasma protein (BPP), porcine plasma protein (PPP), and chicken plasma protein (CPP) decreased the whiteness, whereas prevented the degradation of proteins in modori gels. The hemolysis of plasma samples is a common problem, especially in the large scale collection of plasma. However, the addition of RC increased the whiteness of modori gel. After addition of RC, lightness (L*) of modori gel increased due to formation of more ordered network during gelation, thus the whiteness increased. Further addition of RC resulted in increase in whiteness in modori gel ($P < 0.05$) (Table 2). The increase in whiteness of modori gel with RC is an additional advantage of RC. The decrease in whiteness was the main disadvantage of mammalian plasma protein in surimi industry, even though it was effective in preventing the autolysis in modori gel with low cost.

Autolysis of surimi gel resulted in irreversible destruction of gel texture and increased simultaneously the expressible drip, an indicative of water holding capacity, of modori gel (Table 2). The highest expressible drip was obtained in modori gel without RC addition. Addition of RC resulted in the decrease in

expressible drip. Expressible drip decreased as the amount of RC increased ($P<0.05$). Protein matrix formed during thermal gelation of modori gel imbibes water throughout the network. Therefore, RC inhibited the autolysis in muscle proteins and resulted in the ordered network formation with high water holding capacity.

바. Effect of RC on the protein degradation of surimi gels

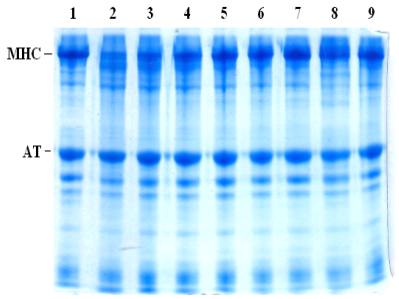

Fig. 5. Protein pattern of surimi gel added with different concentrations of recombinant cystatin and controls. MHC: myosin heavy chain; AC: actin. 1: surimi gel directly heated; 2: surimi gel without inhibitor; 3–7: surimi gel set with addition of 10, 30, 60, 100, 150 g/g recombinant inhibitor, respectively; 8: surimi gel with addition of 60 g/g chymostatin, 9: surimi get with addition of 2% egg white powder.

Myosin heavy chain (MHC) withthe molecular weight (Mw) of 200 kDa was found to be the major protein in surimi, and actin was the second abundant protein in surimi with a Mw of 45 kDa. Cathepsin B, L, and other heat stable proteases active at around 55 ℃cause rapid and severe degradation of myofibrillar proteins, particularly myosin. The addition of RC inhibited the degradation of MHC in modori gel (Fig. 5). With the addition of RC, TCA-soluble peptides derived mainly from the degradation of myofibrillar proteins in surimi decreased (Figure 3). The increase of RC addition resulted in more MHC retained, but had no effect on actin. It's reported that mainly the myosin acted as a substrate for myofibril-bound protease and actin was rather stable, which was the same as the result in this study. EW was effective in preventing the degradation of MHC, but more protein bands with molecular weight less than 200 kDa could be observed (Figure 5), and the band intensity of MHC was lower than that of RC at 100 and 150 μg/g. Therefore, RC could effectively inhibit the degradation of MHCin modori gel better than EW, and the

inhibition was in a concentration-dependent matter.

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제 9절 화장품 및 젓갈 관련 단백분해효소 저해활성 비교 효소 저해활성

1. Inhibition of elastase

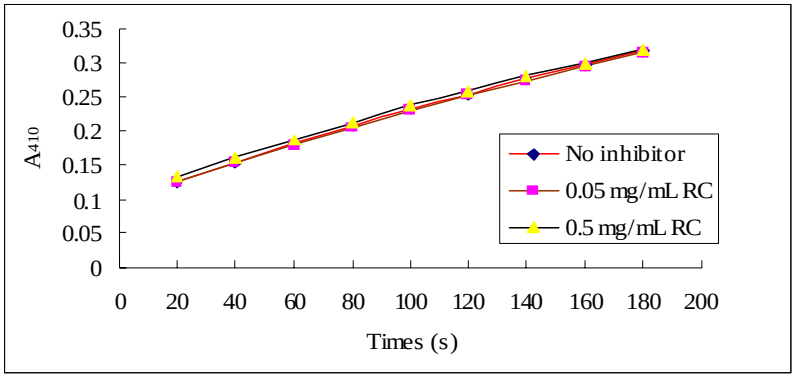


Fig.1. The inhibition of elastase at different ocncentration of R. C.

No obvious inhibitory activity against elastase was checked at two

concentrations of recombinant cystatin.

2. Inhibition of collagenase

Table 1. The inhibition of collagenase at different concentration of R.C.

	Recombinant cystatin	
Concentrarion (mg/mL)	0.05	0.20
Inhibitory activity (U/mg)	0.04	-

At 0.05 mg/mL, the recombinant cystatin showed some inhibitory activity against collagenase from Clostridium histolyticum (Table 1). As the concentration increased to 0.2 mg/mL, no inhibitory activity could be detected.

3. Inhibition of tyrosinase

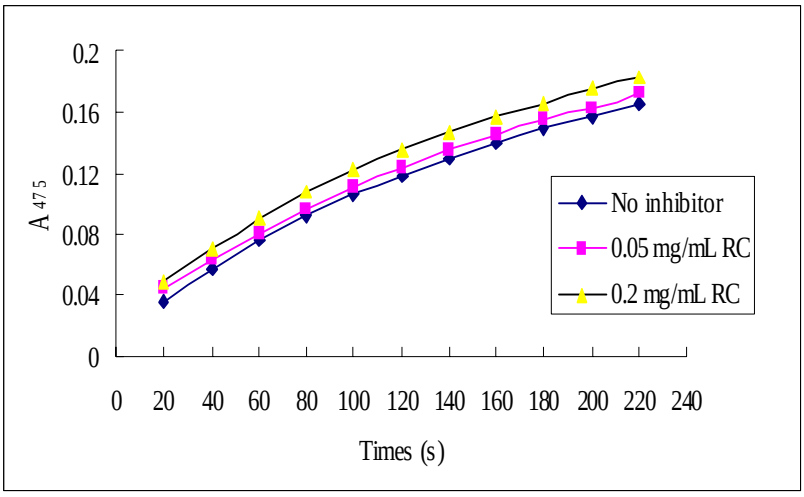


Fig. 2. The inhibition of tyrosinase at different concentration of R.C.

No obvious inhibitory activity against mushroom tyrosinase was determined at two different concentrations of recombinant cystatin.

제 10절 송어 혈장 단백질의 특성

1. Effect of fish plasma on textural properties of surimi gel

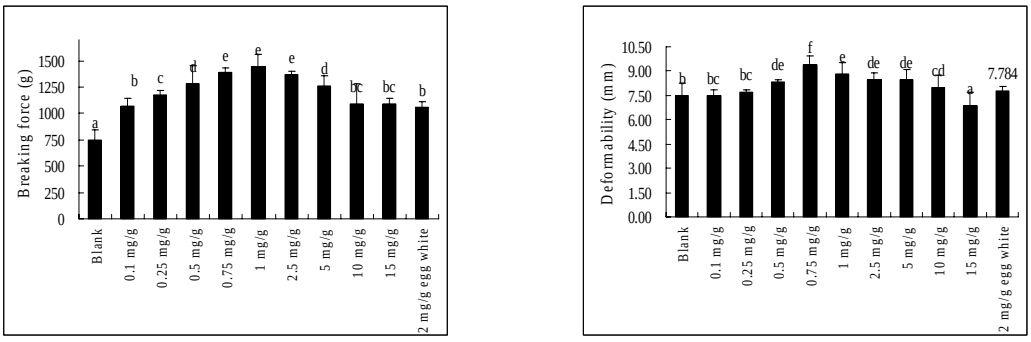


Fig. 1. Breaking force and deformation of surimi gels at different concentrations of fish plasma. Bars represent the standard deviation from five determinations. Different letters in the same grade surimi indicate significant differences ($p < 0.05$).

Surimi gel at 1 mg/g of fish plasma showed the highest breaking force which was around twice of the blank. At addition of 0.75 mg/g, the highest deformation was obtained. The breaking force and deformation were both higher than those of the addition of egg white with 2 mg/g.

2. Effect of fish plasma on whiteness and expressible moisture of surimi gels

Table 1. Whiteness of surimi gels at different concentrations of fish plasma

sample	whitness
blank	51.19 ± 0.72^{c}
0.1 mg fish plasma	51.94 ± 0.81^{de}
0.25 mg/g fish plasma	51.98 ± 0.50^{de}
0.5 mg.g fish plasma	52.79 ± 0.47^{de}
0.75 mg/g fish plasma	53.72 ± 0.20^{f}
1 mg/g fish plasma	51.16 ± 0.12^{c}
2.5 mg/g fish plasma	50.36 ± 1.01^{d}
5 mg/g fish plasma	50.17 ± 0.32^{a}
2 mg/g egg ehite	51.65 ± 0.22^{d}

Values in the table are mean ± SD from triplicate determinations.
abcdef: Different superscripts in the same column indicate significant differences ($p < 0.05$)

Table 2. Expressible moisture of surimi gels at different concentrations of fish plasma

sample	whitness
blank	17.85 ± 3.29^{g}
0.1 mg fish plasma	11.93 ± 0.69^{e}
0.25 mg/g fish plasma	9.98 ± 1.3^{cd}
0.5 mg.g fish plasma	6.18 ± 1.36^{a}
0.75 mg/g fish plasma	7.63 ± 0.94^{b}
1 mg/g fish plasma	8.65 ± 1.99^{f}
2.5 mg/g fish plasma	11.01 ± 1.02^{de}
5 mg/g fish plasma	9.33 ± 1.79^{c}
2 mg/g egg ehite	11.46 ± 0.17^{de}

Values in the table are mean ± SD from triplicate determinations.
abcdefg: Different superscripts in the same column indicate significant differences ($p < 0.05$)

Texture and color were main effectors determining the market value of surimi-based products. With the addition of fish plasma, the whiteness of surimi gel increased, which was consistent with the decrease of expressible moisture (Table 2). Fish plasma at 0.75 mg/g showed the highest whitening effect, Further addition would decrease the whiteness. The effect of fish plasma was better than that from 2 mg/g of egg white powder addition.

3. Effect of fish plasma on protein degradation in surimi gels

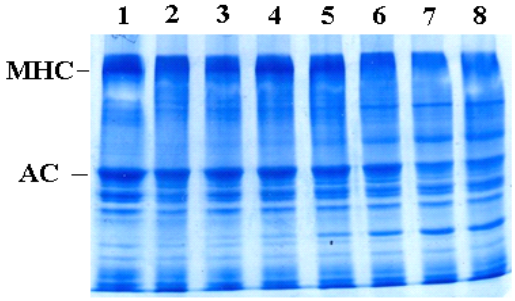


Fig. 3. Protein pattern of surimi gel at different concentrations of fish plasma. MHC, myosin heavy chain; AC, actin; 1, directly heated surimi gel; 2, surimi gel without inhibitor; 3-8, surimi gel with addition of 0.5, 0.75, 1.0, 2.5, 5.0 and 10.0 mg/g fish plasma.

Below 0.75 mg/g, the fish plasma could inhibit the degradation of myosin heavy chain, further addition will increase the degradation of MHC.

제 4 장 단백분해효소 저해제의 대량 생산 및 생산

공정의 최적화

제 1 절 김 (홍조류) 및 파래 (녹조류)에서 단백질분해효소 저해제 정제 및 활성 연구

1. 김 유래 단백분해효소 저해제

Ammonium sulfate에 농도 0-20%에서의 단백질의 양은 45 mg으로 타 구간에 비하여 월등히 높았으나 specific inhibitory activity는 0.04 unit/mg으로서 타 구간에 비해 매우 낮았다 (Table 1). Specific inhibitory activity는 40-60% 구간에서 약 16 unit/mg으로 가장 높게 측정되었고 80-100% 구간에서는 저해활성이 나타나지 않았다. 따라서 40-60% 구간의 단백질을 이용하여 이온교환 크로마토그래피를 수행하려고 하였으나, 2 kg의 김을 이용하였음에도 불구하고 이 구간에서 추출된 단백질의 양이 0.14 mg에 불과하였으므로, total inhibitory activity의 yield가 가장 높은 20-40% 구간 및 60-80% 구간의 단백질까지 모두 모아 함께 이온교환 크로마토그래피를 수행하였다.

Table 1. Ethanol로 추출된 방사무늬김 단백질 함량과 추출된 단백질의 papain 저해활성

Ammonium sulfate concentration fraction (%)	Concentration of total protein (μg/ml)	Amount of total protein (mg)	Total inhibitory activity (unit)	Specific inhibitory activity (unit/mg)	Yield[a] (%)
No treatment	798.47	139.73	19.25	0.13	100
0-20	600	45	2.1	0.04	10.90
20-40	8.85	0.66	4.2	6.32	21.81
40-60	1.98	0.14	2.4	16.12	12.46
60-80	2.74	0.20	2.775	13.46	14.41
80-100	1.98	0.14	0	0	0

a yield = 100(total inhibitory activity in sample/total inhibitory activity obtained for no treatment of ammonium sulfate)

물과 sonication을 이용한 기계적 추출방법은 ethanol 추출의 경우에 비하여 단백질 추출이 훨씬 효과적이었으며 ammonium sulfate fraction 전 구간에 걸쳐 고르게 단백질이 추출 되었다(Table 2). Specific inhibitory activity는 60-80, 80-100%의

구간에서는 나타나지 않았으나, 나머지 구간에서는 ethanol 추출 시와 비슷하였다. 특히 구간 20-40%의 경우, 회수된 단백질 총량, specific inhibitory activity, total inhibitory activity, yield 등 모든 값들이 가장 높게 나타났다.

Table 2. 물과 sonication을 이용한 기계적 방법으로 추출된 방사무늬 김의 단백질 함량과 추출된 단백질의 papain 저해활성

Ammonium sulfate concentration (%)	Concentration of total protein (μg/ml)	Amount of total protein (mg)	Total inhibitory activity (unit)	Specific inhibitory activity (unit/mg)	Yield[a] (%)
No treatment	66.87	80.24	1560	19.44	100
0-20	241.22	18.09	150	8.29	9.61
20-40	256.48	19.24	300	15.59	19.23
40-60	56.94	4.27	52.5	12.29	3.36
60-80	218.32	16.37	0	0	0
80-100	101.98	7.65	0	0	0

a. yield = 100(total inhibitory activity in sample/total inhibitory activity obtained for no treatment of ammonium sulfate)

가장 높은 inhibitory activity는 약 0.09 unit 이었고 이는 단백질 peak의 오른쪽 부분에서 측정되었다 (Fig. 1). 이는 사용된 단백질 중 상대적으로 (+) charge를 많이 함유한 단백질들이 papain 저해력이 뛰어나다는 것을 의미한다. 본 실험의 결과와 cystatin에 대한 기존 보고를 고려하여 볼 때, 비록 직접적이지는 않으나 단백질의 (+) charge가 papain과 같은 단백질분해효소를 저해하는데 특정한 역할을 한다는 것을 추론해볼 수 있다.

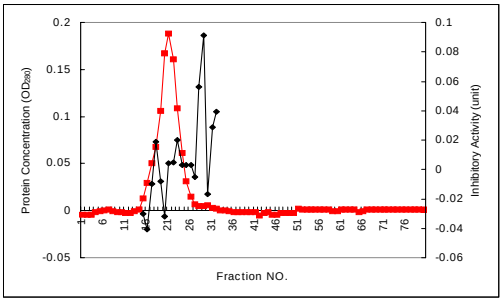


Fig. 1. 이온교환 크로마토그래피에 의한 방사무늬 김의 단백질 분리 및 분리된 단백질의 papain 저해활성.

이온교환 크로마토그래피에서 분리된 fraction 28과 29를 모아 전기영동을 실시하였다(Fig. 2). . 명확한 단백질 band는 66 KDa보다 약간 큰 부근에서 관찰되었으며, 이는 일반적으로 알려진 cystatin의 분자량 약 13 KDa (Barrett, 1981) 보다 훨씬

크다. 이 후 35 KDa 부근에서 희미한 band가 관찰되었으나 이것이 단백질 band인지는 아직 명확하지 않다. 본 실험결과로 볼 때, 방사무늬김의 ethanol extract에 존재하는 단백질분해효소 저해제는 (+) charge를 다량 함유하며 분자량은 66 KDa보다 약간 커 일반적인 egg white cystatin 보다는 훨씬 큰 단백질이라고 예상된다.

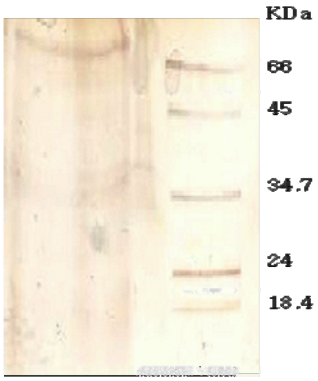


Fig. 2. 전기영동에 의한 방사무늬김의 단백질 분리

2. 파래 유래 단백질분해효소 저해제

측정된 단백질 양과 inhibitory activity는 김의 ethanol extract의 경우와 매우 다른 양상을 보여주었다 (Table 3). 우선 단백질은 precipitation fraction이 0-20, 20-40%인 구간에서 각각 약 12 mg으로서 상대적으로 다량 검출되었고 나머지 구간에서는 0.5 mg 이하의 미량만이 검출되었다. 그러나 0-20, 20-40% 구간에서의 specific inhibitory activity는 각각 약 0.1 unit/mg 으로서 매우 낮았다. 구간 80-100%의 결과를 보면, 이 구간에서는 비록 0.03 mg 정도로 미량의 단백질만이 추출되었으나 이 구간의 inhibitory activity는 약 21 unit/mg로서 다른 구간에 비하여 월등히 높았다. 따라서 이 구간만을 이용하여 이온교환 크로마토그래피를 수행하였다.

Table 3. Ethanol로 추출된 창자파래의 단백질 함량과 추출된 단백질의 papain 저

해활성

Ammonium sulfate concentration (%)	Total concentration of protein (μg/ml)	Total amout of protein (mg)	Total inhibitory activity (unit)	Specific inhibitory activity (unit/mg)	Yield[a] (%)
No treatment	124.88	24.97	10.1	0.40	100
0-20	485.49	12.13	1.425	0.11	14.10
20-40	462.59	11.56	1.3125	0.11	12.99
40-60	12.48	0.31	1.1375	3.64	11.26
60-80	1.57	0.03	0.125	3.17	1.23
80-100	1.34	0.03	0.7125	21.21	7.05

a. yield = 100(total inhibitory activity in sample/total inhibitory activity obtained for no treatment of ammonium sulfate)

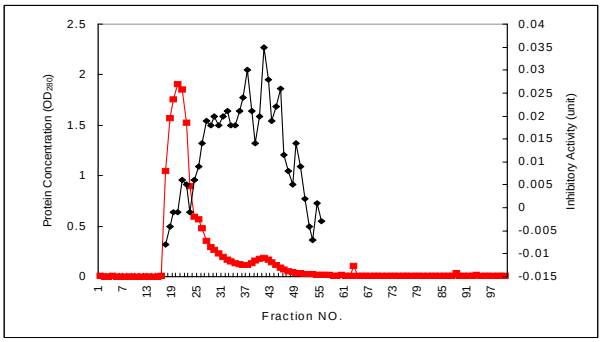


Fig. 3. 이온교환크로마토그래피에 의한 창자파래의 단백질 분리 및 단백질의 papain 저해활성.

이온교환 크로마토그래피에서는 2개의 단백질 peak가 관찰되었으며 retention time이 보다 긴 쪽에서 관찰된 작은 단백질 peak의 inhibitory activity가 상대적으로 높았다 (Fig. 3).

제 2 절 해조 추출물의 단백분해효소 저해효과 및 해조유래 저해제와의 특성비교

1. 실험재료 및 방법

가. 실험재료

김으로는 원산지가 부산 기장인 방사무늬김 (*Porphyra yezoensis*)을 사용하였으며, 파래로는 원산지가 부산 기장인 창자파래 (*Enteromorpha intestinalis*)를 사용하

였다. 자연산 방사무늬김과 창자파래를 실온에서 건조시킨 후 영하 40℃ 냉동고에 보관한 후 실험에 사용하였다.

나. 실험방법

(1) 단백질 저해제 추출

단백질 저해제 추출은 Yermakova et al. (2002)의 방법을 수정하여 수행하였다. 냉동고의 김 (2000 g)과 파래 (400 g)를 실온에서 물에 불려 물기를 짜낸 후 각각 ethanol (1:1, w/w) 용액에 넣고 blender를 이용하여 균질화 한 후 실온에서 2일간 방치하였다. 균질액을 CHCl3를 이용하여 처리한 후 CHCl3 층을 분리, 제거하고, 남아있는 ethanol fraction을 진공을 이용하여 농축하였다. 여기에 80% ethanol을 첨가하고 간단히 교반한 후 진공에서 ethanol을 증발시킨 후 동결건조하여 고형물을 회수하였다. 고형물을 단계적으로 물을 첨가하여 완전히 녹인 후 10% (w/w) PVPP (polyvinyl polypyrrolidone, Sigma Chemical Co., St. Louis, Mo)를 첨가하고 20분간 교반한 후 원심분리하여 sample에 잔존하는 색소를 제거하였다.

5개의 원심분리 셀에 각각 sample 100ml을 넣고 ammonium sulfate를 첨가하여 precipitation fraction을 각각 0-20%, 20-40%, 40-60%, 60-80%, 80-100%로 하였다. 이들을 원심분리하여 침전물을 회수한 후 50 mM sodium acetate buffer에 녹이고 ammonium sulfate의 포화도에 따른 sample의 단백질 양과 단백질분해효소 저해활성 (protease inhibition activity)을 측정하였다.

김의 경우, ethanol을 사용하지 않고 물을 이용한 기계적 추출도 병행하였는데, 이때는 불린 김을 다시 실온에서 2일간 건조하여 최종 중량 50 g 으로 한 후 사용하였다. 건조된 김을 blender를 이용하여 분쇄한 후 300 μm sieve를 이용하여 거르고 적정량의 물을 첨가하면서 막자사발을 이용하여 갈았다. 이 후 약 500 ml의 물을 첨가한 후 1시간 동안 sonication 하고 원심분리하여 supernatant를 회수하였다. 회수된 supernatant에 10% (w/w) PVPP를 첨가하고 20분간 교반한 후 원심분리하여 sample에 잔존하는 색소를 제거하였다. 이 후 위에서 기술된 바와 같이 서로 다른 ammonium sulfate precipitation fraction을 이용하여 단백질을 회수하였다.

(2) 단백질 정량

Ammonium sulfate precipitation fraction에 따른 단백질의 양은 Bio-Rad Protein Kit (Bio-Rad Laboratories, Hercules, CA)을 이용하여 측정하였다. Sample 200 μl에 Bio-Rad protein assay reagent A 100 μl를 첨가한 후 10분간 방치하였다. 그 후 Bio-Rad protein assay reagent B 800 μl를 다시 첨가한 후 15 분간 방치하고 적절하게 희석한 후 700 nm에서 흡광도를 측정하여 standard curve를

이용하여 단백질 함량을 계산하였다. Standard curve는 albumin을 0, 5, 10, 15, 20 μg/ml의 농도로 물에 녹여 흡광도를 측정하여 준비하였다.

(3) 단백질분해효소 저해활성 측정

추출된 단백질의 단백질분해효소 저해활성은 papain과 azo-casein의 반응을 기본으로 한 Weerasinghe, et al. (1996)과 Borla et al. (1998)의 방법을 수정하여 사용하였다. Sample 200 μl를 80 ℃에서 10분간 가열한 후 여기에 100 μl papain 용액 을 첨가하였다. Papain 용액은 papain (Sigma Chemical Co.)을 0.5 mg/ml의 농도로 pH 6.0의 sodium phosphate buffer에 녹여 준비하였다. Control로서는 sample 대신 pH 5.5의 sodium acetate buffer 200 μl를 이용하였다. 각 sample의 blank를 만들기 위하여 20% (w/v) TCA (trichloroacetic acid, Sigma Chemical Co.)용액을 700μl 첨가하여 papain을 불활성화 시켰다. 37 ℃ 항온수조에서 5분간 방치한 후 papain의 substrate인 azo-casein 용액 250 μl를 첨가하였다. Azo-casein 용액은 2 mg의 azo-casein (Sigma Chemical Co.)을 0.625 ml 증류수에 녹여 준비하였다. 37 ℃ 항온수조에서 30 분 방치한 후 blank 이외의 실험구간에 700μl TCA 용액을 첨가하여 papain 효소반응을 정지 시켰다. Sample을 원심분리한 후 supernatant와 1 N NaOH용액의 비율을 9:10 (v/v)으로 하여 440 nm에서의 흡광도 (OD440)를 측정하였다. Papain 활성 1 unit는 1 cm 큐벳을 이용하였을 경우 주어진 조건 (0.05 mg papain, 30분 반응시간)에서 측정되는 흡광도로서 정의하였다. 저해활성 1 unit는 papain 활성 1 unit 감소로 정의하였다.

(4) 이온교환 크로마토그래피

추출된 단백질 양과 추출된 단백질의 specific papain 저해활성 (inhibitory activity (unit)/단백질의 양 (mg))을 동시에 고려하여 적절한 ammonium sulfate 포화도 구간을 선택한 후 그 sample 단백질을 이온교환 크로마토그래피를 이용하여 분리하였다. Sample에 존재하는 저분자 물질을 제거하기 위하여 dialysis를 먼저 실시하였다. Sample 약 15 ml를 cellulose membrane (width = 18 ± 2 mm, diameter = 11.5 mm, capacity = approx. 1.0ml/cm, Spectrum Labs Com., Rancho Dominguez, CA)으로 만들어진 튜브에 넣어 pH 5.5의 50 mM sodium acetate buffer에 침지한 후 4 ℃에서 over night하였다.

이온교환 크로마토그래피는 Pharmacia Biotech (1995)의 방법을 기본으로 하여 CM-Sepharose column (Pharmacia Biotech, Uppsala, Sweden)을 이용하여 수행하였다. Column은 pH 5.5의 50 mM sodium acetate buffer로 equilibrate 하였다. Sample을 column에 주입한 후 50 mM sodium acetate buffer와 0.1 M NaCl 이 함

유된 50 mM sodium acetate buffer를 이용하여 NaCl gradient 조건에서 sample을 elution 하면서 fraction collector를 이용하여 분리된 sample을 수거하였다. 수거된 각 factrion의 흡광도를 280 nm에서 측정하여 상대적인 단백질의 양을 분석하였다. 상대적인 단백질의 함량이 높은 fraction에서 papain 저해활성을 위에서 기술된 방법을 이용하여 측정하였다. 단 sample을 가열하는 과정은 생략하였다.

(5) 전기영동

상대적인 papain 저해활성이 큰 faction을 결정한 후, 이 fraction의 단백질 종류와 크기를 결정하기 위하여 Yamashita and Konagaya (1991)의 방법을 이용하여 SDS-PAGE (sodium dodecyl sulfate polyacrylamide gel electrophoresis, 8.0 × 7.3 cm)를 수행하였다. 단백질 marker로서 Sigma Chemical Co.로부터 구입한 bovin albumin (66,000 Da), egg albumin (45,000 Da), pepsin (34,700 Da), trypsinogen (24,000 Da), β lactoglobulin (18,400 Da)의 혼합물을 사용하였다. 단백질의 염색은 Bio-Rad Laboratories의 silver stain kit를 이용하여 수행하였다.

2. 연구결과

가. 김 유래 단백질분해효소 저해제

방사무늬 김으로부터 단백질분해효소 저해제로 작용하는 단백질을 분리하기 위하여 우선 ethanol을 이용한 추출을 실시하였다. Ethanol 추출은 Yermakova et al. (2002)이 해양 연체동물의 endo-(1→3)-β-D-glucanases의 활성을 저해하는 단백질을 해조류중의 하나인 Laminaria cichorioides에서 추출하기 위하여 사용한 방법이다. Ethanol extract에 함유된 단백질을 total charge에 따라 분리하기 위하여 ammonium sulfate를 이용하여 침전시키고 각 precipitation fraction에 따라 침전된 단백질의 양과 이들의 papain 저해활성 (inhibitory activity)을 측정하였다 (Table 1). Precipitation fraction이 0-20%인 구간에서는 상대적으로 hydrophobic 하거나 혹은 크기가 작은 (total charge수가 작은) 단백질을 침전시키는데, 이 실험의 경우 추출된 단백질의 양은 45 mg으로 타 구간에 비하여 월등히 높았으나 specific inhibitory activity는 0.04 unit/mg으로서 타 구간에 비해 매우 낮았다. 이는 이 구간에서는 inhibitor로서 작용할 수 있는 단백질의 양이 매우 작거나 혹은 오히려 activator로서 작용할 수 있는 물질들이 함유되어 있음을 의미한다. 0-20% 구간을 제외한 나머지 구간에서는 1 mg 이하의 소량의 단백질만이 추출되었다. Specific inhibitory activity는 40-60% 구간에서 약 16 unit/mg으로 가장 높게 측정되었고 80-100% 구간에서는 저해활성이 나타나지 않았다. 따라서 40-60% 구간의 단백질

을 이용하여 이온교환 크로마토그래피를 수행하려고 하였으나, 2 kg의 김을 이용하였음에도 불구하고 이 구간에서 추출된 단백질의 양이 0.14 mg에 불과하였으므로, total inhibitory activity의 yield가 가장 높은 20-40% 구간 및 60-80% 구간의 단백질까지 모두 모아 함께 이온교환 크로마토그래피를 수행하였다.

Table 1. Ethanol로 추출된 방사무늬김 단백질 함량과 추출된 단백질의 papain 저해활성

Ammonium sulfate precipitation fraction (%)	Concentration of total protein (μg/ml)	Amount of total protein (mg)	Total inhibitory activity (unit)	Specific inhibitory activity (unit/mg)	Yield[a] (%)
No treatment	798.47	139.73	19.25	0.13	100
0-20	600	45	2.1	0.04	10.90
20-40	8.85	0.66	4.2	6.32	21.81
40-60	1.98	0.14	2.4	16.12	12.46
60-80	2.74	0.20	2.775	13.46	14.41
80-100	1.98	0.14	0	0	0

a. yield = 100(total inhibitory activity in sample/total inhibitory activity obtained for no treatment of ammonium sulfate)

물과 sonication을 이용한 기계적 추출방법을 사용하였을 경우, ethanol 추출을 사용하였을 경우와는 매우 다른 결과가 관찰되었다 (Table 2). 우선 ethanol 추출의 경우에 비하여 단백질 추출이 훨씬 효과적이었으며 ammonium sulfate precipitation fraction 전 구간에 걸쳐 고르게 단백질이 추출 되었다. Specific inhibitory activity는 60-80, 80-100%의 구간에서는 나타나지 않았으나, 나머지 구간에서는 ethanol 추출 시와 비슷한 값과 측정되었다. 특히 구간 20-40%의 경우, 회수된 단백질 총량, specific inhibitory activity, total inhibitory activity의 yield 등의 모든 값들이 가장 높게 나타났다. 현재 구간 0-20, 20-40, 40-60%에서 얻어진 단백질을 각각 이온교환 크로마토그래피를 이용하여 분리하는 과정에 있다.

Table 2. 물과 sonication을 이용한 기계적 방법으로 추출된 방사무늬 김의 단백질

함량과 추출된 단백질의 papain 저해활성

Ammonium sulfate precipitation fraction (%)	Concentration of total protein (μg/ml)	Amount of total protein (mg)	Total inhibitory activity (unit)	Specific inhibitory activity (unit/mg)	Yield[a] (%)
No treatment	66.87	80.24	1560	19.44	100
0-20	241.22	18.09	150	8.29	9.61
20-40	256.48	19.24	300	15.59	19.23
40-60	56.94	4.27	52.5	12.29	3.36
60-80	218.32	16.37	0	0	0
80-100	101.98	7.65	0	0	0

a. yield = 100(total inhibitory activity in sample/total inhibitory activity obtained for no treatment of ammonium sulfate)

Ethanol 추출 후 ammonium sulfate precipitation fraction이 20-40, 40-60, 60-80% 인 구간을 모아 이온교환 크로마토그래피를 수행하고, 단백질 peak가 관찰된 부분을 중심으로 하여 papain에 대한 inhibitory activity를 측정하였다 (Figure 1). 가장 높은 inhibitory activity는 약 0.09 unit 이었고 이는 단백질 peak의 오른쪽 부분에서 측정되었다. 이는 사용된 단백질 중 상대적으로 (+) charge를 많이 함유한 단백질들이 papain 저해력이 뛰어나다는 것을 의미한다. Tsai and Chang (1996)은 양이온교환 크로마토그래피를 실시하였을 때, 다른 단백질에 비하여 긴 retention time을 가지는, 즉 상대적으로 (+) charge를 많이 함유한, 단백질 부분에 단백질분해효소 저해제로 잘 알려진 cystatin류가 존재한다는 것을 보고하였다. 또한 Barrett (1981)은 cystatin류가 alkali에 안정하다고 하였는데 이 역시 cystain이 다량의 (+) charge를 가지고 있음을 간접적으로 시사한다. 본 실험의 결과와 cystatin에 대한 기존 보고를 고려하여 볼 때, 비록 직접적이지는 않으나 단백질의 (+) charge가 papain과 같은 단백질분해효소를 저해하는데 특정한 역할을 한다는 것을 추론해볼 수 있다. 실험오차의 범위 밖에서 inhibitory activity가 (-) 값을 가지는 faction도 관찰되었는데, 정확한 이유는 알 수 없으나 아마도 이들 fraction에는 papain activator의 역할을 하는 단백질 혹은 다른 물질이 존재할 수도 있다고 추론된다.

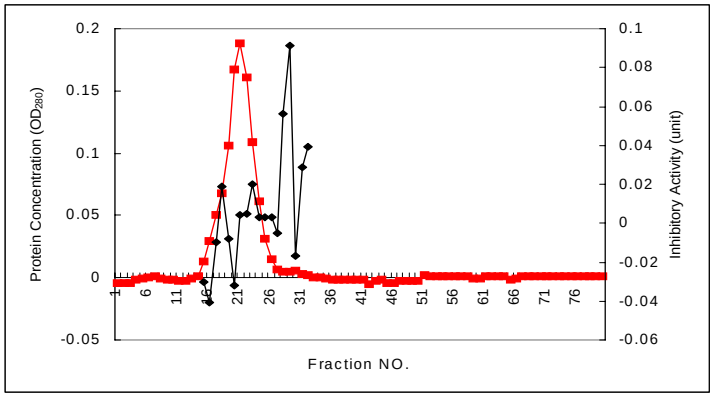


Fig 1. 이온교환 크로마토그래피에 의한 방사무늬 김의 단백질 분리 및 분리된 단백질의 papain 저해활성

이온교환 크로마토그래피에서 분리된 fraction 중 다른 fraction에 비해 inhibitory activity가 0.06, 0.09 unit로서 가장 큰 fraction 28과 29를 모아 전기영동을 실시하여 단백질의 종류와 크기를 알아보았다 (Figure 2). 명확한 단백질 band는 66 KDa보다 약간 큰 부근에서 관찰되었으며, 이는 일반적으로 알려진 cystatin의 분자량 약 13 KDa (Barrett, 1981) 보다 훨씬 크다. 이 후 35 KDa 부근에서 희미한 band가 관찰되었으나 이것이 단백질 band 인지는 아직 명확하지 않다. 본 실험결과로 볼 때, 방사무늬김의 ethanol extract에 존재하는 단백질분해효소 저해제는 (+) charge를 다량 함유하며 분자량은 66 KDa 보다 약간 커 일반적인 egg white cystatin 보다는 훨씬 큰 단백질이라고 예상된다.

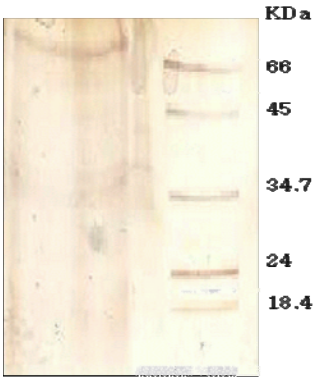


Fig. 2. 전기영동에 의한 방사무늬김의 단백질 분리

나. 파래 유래 단백질분해효소 저해제

창자파래로부터 단백질분해효소 저해제로 작용하는 단백질을 분리하기 위하여 ethanol을 이용한 추출을 실시하였다. 측정된 단백질 양과 inhibitory activity는 김

의 ethanol extract의 경우와 매우 다른 양상을 보여주었다 (Table 3). 우선 단백질은 precipitation fraction이 0-20, 20-40%인 구간, 즉 상대적으로 hydrophobic 하거나 혹은 크기가 작은 (total charge수가 작은) 단백질이 침전되는 구간에서 각각 약 12 mg으로서 상대적으로 다량 검출되었고 나머지 구간에서는 0.5 mg 이하의 미량만이 검출되었다. 그러나 0-20, 20-40% 구간에서의 specific inhibitory activity는 각각 약 0.1 unit/mg 으로서 매우 낮았다. 구간 80-100%의 결과를 보면, 이 구간에서는 비록 0.03 mg 정도로 미량의 단백질만이 추출되었으나 이 구간의 inhibitory activity는 약 21 unit/mg로서 다른 구간에 비하여 월등히 높았다. 따라서 이 구간만을 이용하여 이온교환 크로마토그래피를 수행하였다.

Table 3. Ethanol로 추출된 창자파래의 단백질 함량과 추출된 단백질의 papain 저해활성

Ammonium sulfate precipitation fraction (%)	Concentration of total protein (μg/ml)	Amount of total protein (mg)	Total inhibitory activity (unit)	Specific inhibitory activity (unit/mg)	Yield[a] (%)
No treatment	124.88	24.97	10.1	0.40	100
0-20	485.49	12.13	1.425	0.11	14.10
20-40	462.59	11.56	1.3125	0.11	12.99
40-60	12.48	0.31	1.1375	3.64	11.26
60-80	1.57	0.03	0.125	3.17	1.23
80-100	1.34	0.03	0.7125	21.21	7.05

a. yield = 100(total inhibitory activity in sample/total inhibitory activity obtained for no treatment of ammonium sulfate)

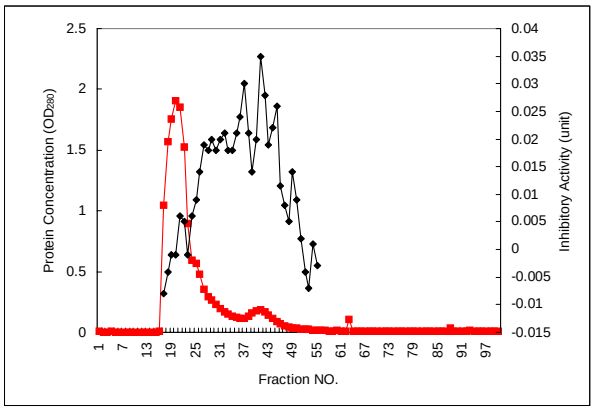


Fig 3. 이온교환 크로마토그래피에 의한 창자파래의 단백질 분리 및 분리된 단백질의 papain 저해활성.

이온교환 크로마토그래피에서는 2개의 단백질 peak가 관찰되었으며 retention time이 보다 긴 쪽에서 관찰된 작은 단백질 peak의 inhibitory activity가 상대적으로 높았다 (Figure 3). 본 실험에서 얻어진 이온교환 크로마토그래피에 의한 단백질 분리 경향은 Tsai and Chang (1996)가 보고한 Carp ovarian fluid의 분석결과와 매우 유사하다. 위 저자의 실험결과에서는 오른쪽 작은 peak에서 약 12 kDa의 cystatin류 inhibitor가 분리, 동정되었다. 현재 작은 peak의 정점부근에서 나타나는 단백질의 종류와 크기를 알아보기 위하여 전기영동을 수행하고 있다.

(1) 해조 추출물의 단백분해효소 저해효과

모든 실험구간에서 water extract의 저해활성과 단백질 함량이 ethanol extract의 값보다 높은 경향을 보여주었다(Table 4). 저해활성과 단백질 함량이 가장 높은 구간은 P. yezoensis의 수용성 추출물 (water extract) 이었는데, 추출된 단백질의 양은 사용한 시료의 1.7% (w/w)로 추출효율이 매우 낮았다.

Table 4. Total protease inhibitory activities and protein contents of water and ethanol extracts from the three seaweeds

Sample	Total inhibitory activity (units) [Protein content (mg)]		
	P. yezoensis	P. tenera	E. intestinalis
Water extract	893.0 [836.3]	622.2 [618.3]	139.2 [250.3]
Ethanol extract	546.3 [150.8]	354.4 [230.1]	10.1 [24.97]

(2) 단백질 저해제의 정제

P. yezoensis의 수용성 추출물에 함유된 단백질을 염석을 통하여 분리하였다 (Table 5). 분리된 단백질의 함량과 total inhibitory activity는 ammonium sulfate 20-40% 구간에서 각각 132 mg, 510 unit로 가장 높았으나 이 구간에서의 specific inhibitory activity는 3.87 unit/mg으로 40-60% 구간의 값 (10.82 unit/mg)보다 훨씬 낮았다. 따라서 단백질의 저해효과가 가장 뛰어난 40-60% 구간에서 침전된 단백질 (10.1배의 purification fold)을 SDS-PAGE를 통하여 분리하여 어떠한 단백질이 단백분해효소 저해에 주요한 영향을 미치는가를 살펴보았다.

Table 5. Purification of protease inhibitors from P. yezoensis using water extraction and salting out

(NH4)2SO4 saturation (%)	Total protein (mg)	Total inhibitory activity (unit)	Specific inhibitory activity (unit/mg)	Yield[a] (%)	Purification fold[b]
Water extract	836.3	893.0	1.07	100	1.0
0-20	42.3	57.0	1.35	6.4	1.3
20-40	131.9	510.0	3.87	57.1	3.6
40-60	6.4	69.0	10.82	7.7	10.1
60-80	6.8	6.6	0.97	0.7	0.9
80-100	10.5	7.8	0.74	0.9	0.7

a Yield of inhibiting fraction = 100(total inhibitory activity/total inhibitory activity of water extract)
b Degree of purification with respect to inhibiting action = specific inhibitory activity/specific inhibitory activity of water extract

SDS-PAGE 결과 10, 66, 78 kDa의 크기를 가지는 세 종류의 단백질이 분리되었다 (Fig. 3). 특히 66 kDa 단백질은 Fig. 2에서 보고 되었던 ion exchange chromatography 후에 얻어진 SDS-PAGE pattern에서도 나타난 바 있는데, 이는 이 크기의 단백질이 단백분해효소 저해에 주요한 역할을 한다는 것을 의미한다.

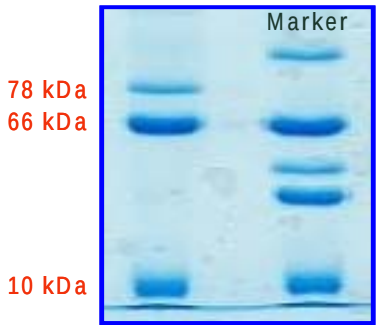


Fig. 3. SDS-PAGE pattern of 40-60% (NH4)2SO4 fraction of water extract

Water extraction에서 추출된 저해제와 ethanol extraction에서 추출된 저해제가 동일한지를 알아보기 위하여 ethanol extract에 함유된 단백질을 ammonium sulfate 염석을 통하여 분리하였다 (Table 6). Water extract의 경우와는 달리 specific inhibitory activity는 80-100% 포화도의 구간에서 10.4 unit/mg으로 가장 높았으므로 이 fraction에 함유된 단백질을 SDS-PAGE를 통하여 분리하였다.

Table 6. Purification of protease inhibitors from P. yezoensis using ethanol extraction and salting out

(NH4)2SO4 saturation (%)	Total protein (mg)	Total inhibitory activity (unit)	Specific inhibitory activity (unit/mg)	Yield[a] (%)	Purification fold[b]
Ethanol extract	150.8	546.3	3.62	100	1.0
0-20	7.2	0	0	0	0
20-40	8.7	2.5	0.29	0.4	0.9
40-60	41.2	0	0	0	0
60-80	2.8	11.3	3.97	2.1	1.1
80-100	5.3	55.0	10.4	10.1	2.9

a Yield of inhibiting fraction = 100(total inhibitory activity/total inhibitory activity of water extract)
b Degree of purification with respect to inhibiting action = specific inhibitory activity/specific inhibitory activity of water extract

SDS-PAGE 결과 10, 66, 78, 100 kDa의 크기를 가지는 네 종류의 단백질이 분리되었는데(Fig. 4), 그 함량이 매우 낮은 100 kDa 단백질을 제외한 나머지 세 단백질의 크기는 water extract의 경우에 관찰되었던 단백질 (Fig. 1)의 크기와 동일하였다. 이는 water 혹은 ethanol extraction에서 추출된 저해제가 같은 종류의 단백질일 가능성이 매우 크다는 것을 의미한다.

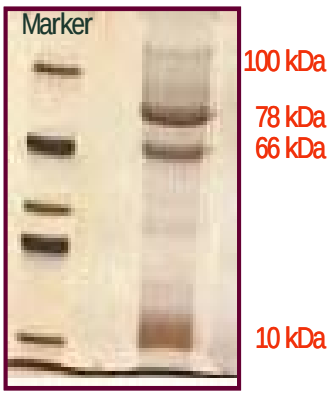


Fig. 4. SDS-PAGE pattern of 80-100% (NH4)2SO4 fraction of ethanol extract

(3) 해조유래 저해제와 농, 축산 저해제와 와의 특성 비교

P. yezoensis 수용성 추출물의 냉동건조 분말 및 수용성 추출물을 ammonium sulfate 40-60% 포화도로 염석한 후 얻어진 단백질의 냉동건조 분말의 저해효과를 cysteine protease인 papain을 이용하여 가장 일반적으로 사용되고 있는 식품용 단백분해효소 저해제인 beef plasma protein, egg white, 그리고 potato powder의 저해효과와 비교평가 하였다.

평가된 모든 저해제의 효과는 높은 온도에서 보다 낮은 온도에서 강하였다 (Fig. 5). 농, 축산 저해제의 경우 모든 온도에서 beef plasma protein의 저해효과가 가장 강하였으며 egg white와 potato powder의 효과는 비슷하였는데 이는 Weerasinghe et al. (1996)의 결과와 일치한다. 냉장온도인 4℃에서는 수용성 해조추출물 분말의 저해효과가 가장 낮게 나타났으나 다른 두 온도에서의 결과로 볼 때 수용성 추출물 분말의 효과는 egg white와 potato powder의 저해효과와 매우 비슷하다고 평가되었다. Ammonium sulfate 40-60% 포화도에서 얻어진 단백질 분말의 저해효과가 모든 온도에서 가장 강하였는데, 이는 저해인자 단백질이 염석에 의하여 고농도로 분리되었기 때문이라 사료된다. 그러나 그 효과는 beef plasma protein의 효과에 비하여 월등하게 뛰어나지 못하였고, water extraction에 의해 얻을 수 있는 단백질의 양이 사용 시료의 0.01%에 불과하므로 본 연구에서 사용된 추출방법을 이용하여 해조로부터 단백분해 저해제를 산업적으로 생산하는 것은 그 경제성이 매우 낮을 것으로 사료된다.

산도를 변화시켰을 경우 모든 저해제에 대하여 pH 4.0에서의 효과가 다른 두 pH에서의 효과보다 약간 높게 나타났지만 전체적인 결과를 고려해 볼 때 pH 4.0 - 6.0에서는 산도가 저해효과에 미치는 영향은 미미하였다(Fig. 6). 실험된 모든 pH 구간에서 수용성 해조추출물 분말의 저해효과는 egg white와 potato powder의 효과와 비슷하였으며 beef plasma protein의 효과보다는 낮았다. 염석에 의하여 얻어진 단백질 분말의 효과가 모든 pH 구간에서 가장 높았으나 beef plasma protein의 효과에 비하여 월등히 큰 값은 보이지 않았다.

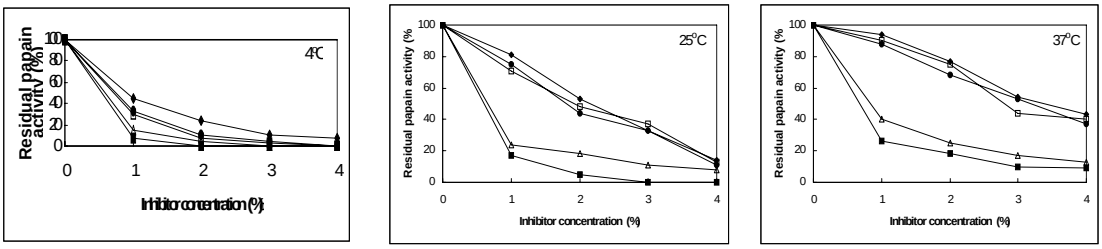


Fig.5. Inhibition of papain action by protease inhibitors at three different temperatures and pH 6.0 (◆, water extract; ■, 40-60% (NH4)2SO4; △, beef plasma protein; ●, egg white; □, potato powder)

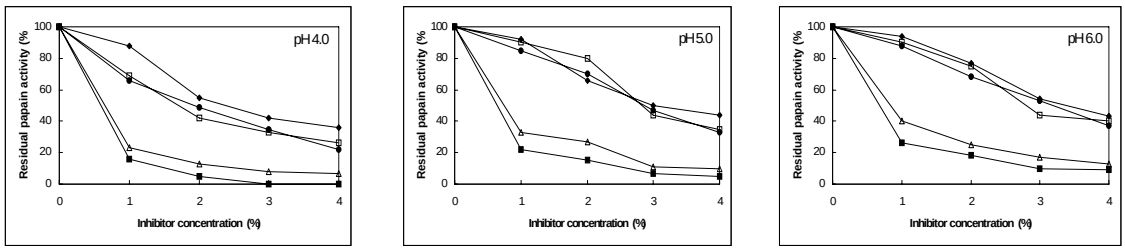


Fig.6. Inhibition of papain action by protease inhibitors at three different pHs 5 and 37℃ (◆, water extract; ■, 40-60% (NH4)2SO4; △, beef plasma protein; ●, egg white; □, potato powder)

제 3 절 *S. cerevisiae*에 의한 재조합 연어 cystatin 생산을 위한 대량발효와 배양공정의 최적화

1. Introduction

Protease inhibitors could be used to prevent the degradation of fish meat and surimi gel. Food grade protease inhibitors commercially used in surimi include whey protein concentrate (WPC), beef plasma protein (BPP), chicken plasma protein (CPP), egg white (EW), and potato extract (PE). However, some side effects such as decrease in whiteness and unpleasant odor were found after their application. Because of the outbreak of bovine spongi form encephalopathy (BSE) and avian influenza (AI), the application of BPP and CPP was limited in surimi industry.

Fish protease inhibitors attract considerable attention after two cystatins were isolated from chum salmon egg. The application of fish protease inhibitors in

surimi gel processing was favorable. Chum salmon cystatin was equipotent to chicken egg white cystatin in the papain inhibitory assay. It was overexpressed in *Saccharomyces cerevisiae* YPH 499, and the recombinant chum salmon cystatin (RC) showed favorable effect on preventing the gel weakening of Alaska pollock surimi.

In order to use fermentor to produce RC on a large scale, the culture condition for growth and induction of recmbinant *S. cerevisiae* was optimized on the basis of former experiments in flask. In flask, galactose was added through changing SC-glucose medium to SC-galactose medium thoroughly by centrifugation before induction. Because of large waste of culture medium and high possibility of contamination, this strategy is not applicable for production of RC in fermentor. Galactose should be directly added into the SC-glucose medium for induction. Thus, the optimization of induction condition for expression of RC was carried out by response surface methodology (RSM) method. Thereafter, the desired combination of aeration and agitation that would yield the highest RC production by *S. cerevisiae* YPH 499 in a 14 L fermentor was studied.

2. Materials and methods

가. Microorganism and media

S. cerevisiae YPH 499 was kept frozen at -80 ℃ in a synthetic minimal defined medium (SC medium) minus uracil containing 20% (w/v) glycerol solution. SC medium minus uracil with a composition of 0.67% yeast nitrogen base (YNB), 2% glucose, 0.01% (adenine, arginine, cysteine, leucine, lysine, threonine, and tryptophan), 0.005% (aspartic acid, histidine, isoleucine, methionine, phenylalanine, proline, serine, tyrosine, and valine) was used for cultivation of recombinant yeast in the flask and fermentor.

나. Experimental design in flask cultivation

For optimization in flask, the experiments were designed using the software, Design Expert (Stat-Easy Co., Minneapolis, MN). The significant independent variables of the cultures are pH of media, inducing time, and inducing assistant amount. The minimum and maximum properties for the mixture design were set

at pH 4.0 to pH 6.0, time from 4 h to 12 h and inducing assistant from 1.4 g/L to 5.6 g/L together with fixed levels of galactose at 20 g/L. (Table 1). Regression analysis was performed on the data obtained from the design experiments.For optimization in fermentor, different agitations and aeration rates were studied with temperature at 30 ℃ and pH 5.7.

다. Cultivation in shake flask

1 vial of frozen *S. cerevisiae* YPH 499 seed was thawed and inoculated into 35 mL of SC medium minus uracil in 300 mL baffled shake flask. After inoculation, the yeast was cultured at 30 ℃ at 150 rpm. During culture, the pH of SC media was adjusted by 1 N NaOH to different values according to RSM design. The content of glucose in SC media was determined by glucose assay kit (Sigma, St. Louis, Mo., USA). After the glucose was excluded, 4 mL of 40% galactose containing different amount of inducing assistant (YNB: amino acids: adenine = 6.7:1.05:0.1) according to RSM design was added to SC media to induce the expression of RC. After different inducing time according to RSM design, 5 mL of SC media was sampled from each of the culture and centrifuged (2000 × g, 5 min). The precipitate was washed with 5 mL of acetate buffer (25 mM sodium acetate containing 1 mM EDTA, 1 mM β-mercaptoethanol, and 50 mM NaCl, pH 6.0) and recentrifuged. The second precipitate was suspended by 1 mL of acetate buffer and used to determine the yield of RC.

라. Cultivation in fermentor

1 vial of frozen *S. cerevisiae* YPH 499 seed was thawed and inoculated into 35 mL of SC medium minus uracil in 300 mL baffled shake flask. After inoculation, the yeast was cultured for 48 h at 30℃nd 150 rpm. Then the culture was inoculated into 250 mL of SC medium minus uracil in 1 L baffled shake flask. The inoculated yeast was cultured for another 6 h and then used as inoculum for fermentor. A 14 L fermentor (Mj-10L, Marubish, JP) containing 7L SC medium minus uracil was used to study the optimal aeration rate and agitation rate. The fermentor was inoculated with the inoculum as described above. The pH was measured using a Mettler Toledo pH electrode immersed into the fermentation broth. Dissolved oxygen (DO) was measured using a

polarographic electrode (Ingold, Leicester, UK). Calibration (the percent of atmospheric oxygen) was performed with air-saturated medium (100%) and nitrogen-saturated medium (0%) after sterilization. Diluted antifoaming agent (KM-70, Hsin-Yu Co., JP) was added when foaming occurred. The pH of the medium was adjusted by 1 N NaOH. Experiments were conducted at agitation speeds of 200, 350, and 500 rpm, respectively. The corresponding aeration rate was adjusted to 0.5, 1.0, and 1.5 vvm (vol. of air/vol. of medium/min), respectively. Samples from the fermentor were drawn at regular intervals and analyzed for cell density and inhibitory activity.

마. Analysis

Cell density and inhibitory activity were determined as described above.

바. Alcoholtreatment of yeast extract

After termination of the fermentor, the culture medium was centrifuged (2000 × g, 10 min, 4 ℃ and the precipitate was washed with acetate buffer (the same volume of the culture medium) and recentrifuged. The second precipitate was added with acetate buffer (one tenth volume of the culture medium), sonicated, and centrifuged (4000 × g, 10 min, 4 ℃. Pre-cooled alcohol solution (95%, -15 ℃ was directly added into the supernatant to final concentration of alcohol at 15%, 30%, 45%, 60%, and 75% (v/v), respectively, and kept at 4 ℃for 2 h. Then the treated yeast extracts were centrifuged (4000 × g, 10 min, 4 ℃. The precipitate was used as crude recombinant chum salmon cystatin and dissolved into acetate buffer or freeze-dried.

3. Results and discussion

가. Fitting the model

Table1. Design matrix of independent variables and their corresponding experimental yields of recombinant chum salmon cystatin

Std	A: pH	B: time	C: inducing assistant	D: galactose	Inhibitory activity
		h	g/L	g/L	U/mL
1	4	4	1.4	20	0.431875
2	6	4	1.4	20	0.33075
3	4	12	1.4	20	0.499673
4	6	12	1.4	20	0.33801
5	4	4	5.6	20	0.31675
6	6	4	5.6	20	0.565875
7	4	12	5.6	20	0.329961
8	6	12	5.6	20	0.529064
9	3.32	8	3.5	20	0.367125
10	6.68	8	3.5	20	0.33525
11	5	1.27	3.5	20	0.48275
12	5	14.73	3.5	20	0.530632
13	5	8	-0.0316	20	0.575867
14	5	8	7.0316	20	0.521008
15	5	8	3.5	20	0.531167
16	5	8	3.5	20	0.536167
17	5	8	3.5	20	0.526167
18	5	8	3.5	20	0.538667
19	5	8	3.5	20	0.523667
20	5	8	3.5	20	0.532067

Table 2. Diagnostics Case Statistics

Standard Order	Actual Value	Predicted Value	Residual	Leverage
1	0.431875	0.476414	-0.04454	0.669768
2	0.33075	0.345607	-0.01486	0.669768
3	0.499673	0.548048	-0.04837	0.669768
4	0.33801	0.36196	-0.02395	0.669768
5	0.31675	0.330512	-0.01376	0.669768
6	0.565875	0.555213	0.010662	0.669768
7	0.329961	0.352817	-0.02286	0.669768
8	0.529064	0.522237	0.006827	0.669768
9	0.367125	0.308286	0.058839	0.607303
10	0.33525	0.340755	-0.00551	0.607303
11	0.48275	0.463771	0.018979	0.607303
12	0.530632	0.496278	0.034354	0.607303
13	0.575867	0.515727	0.060141	0.607303
14	0.521008	0.527814	-0.00681	0.607303
15	0.531167	0.532842	-0.00168	0.16634
16	0.536167	0.532842	0.003325	0.16634
17	0.526167	0.532842	-0.00668	0.16634
18	0.538667	0.532842	0.005825	0.16634
19	0.523667	0.532842	-0.00918	0.16634
20	0.532067	0.532842	-0.00078	z0.16634

In a previous study one factor optimization was considered, and the appropriate range of critical factors contributing to the increased production of yield was selected, which is the SC medium, inducing pH and inducing time. In this study, the SC medium was still used on the basis of plasmid stability of *S. cerevisiae* YPH 499. RSM is used to determine the optimal response of *S. cerevisiae* YPH 499for the expression of RC under a wide range of nutrient conditions. A full factorial central composite experimental design was used to obtain the combination of values that optimizes the response within the region of three dimensional observation spaces, which allows one to design a minimal number of experiments. Because galactose was directly added to SC medium for induction, inducing pH, inducing time, and inducing assistant amount were considered for RSM study to maximize the RC yield in *S. cerevisiae* YPH 499. The design matrix in actual terms and the experimental results of RCinhibitory activity from *S. cerevisiae* YPH 499 are shown in Table 1. Different combinations of inducing pH, inducing time, and inducing assistant amount yielded RC as low as 0.31675 U/mL and as high as 0.575867 U/mL. The predicted values are listed in Table 2. Applying multiple regression analysis, the results were fitted to a quadratic equation. Thus, the mathematical regression model for RC yield fitted in terms of coded factors was obtained as follows:

Activity = -0.91 + 0.63 pH + 0.044 × time -7.678E-03 × inducing assistant - 0.074 × pH2 - 1.167E-03 × time2 - 1.420E-06 × inducing assistant2 - 3.455E-03 × pH × time + 1.693E-03 × pH × inducing assistant - 5.873E-05 × time × inducing assistant

나. Optimization of process

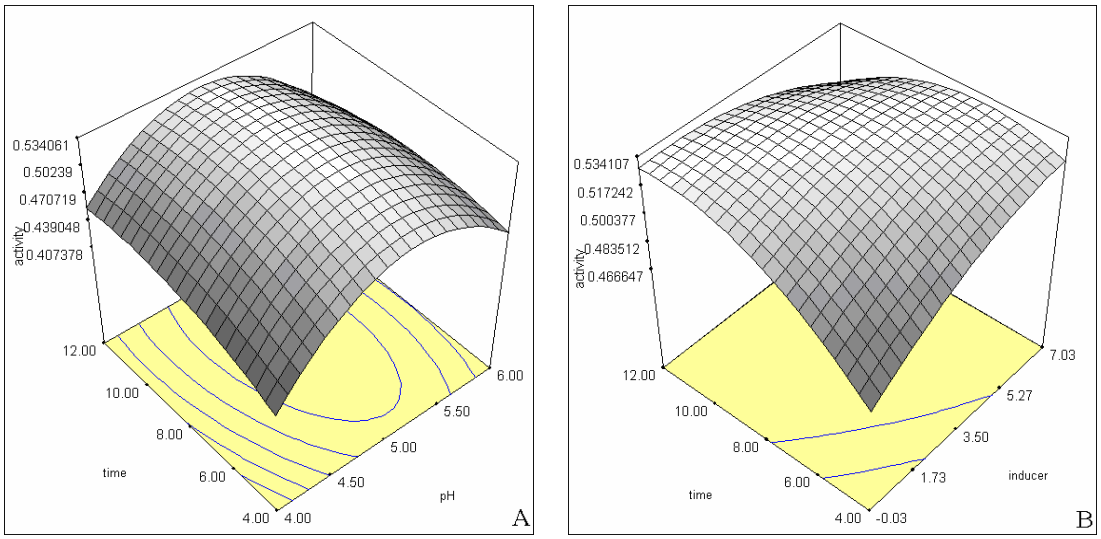


Fig.1. 3D response surface: Interactive effects of varied inducing time (h) and inducing pH at 3.5 g/L of inducing assistant (A); Interactive effects of inducing time (h) and inducing assistant amount (g/L) at pH 5.00 (B).

RC yields from different levels of the variables were predicted from the respective contour plots (Fig. 1. A-B). Each contour curve represents an infinite number of combinations of two test variables with another one maintained at its respective zero levels. Elliptical nature of the contour in 3D-response surface graphs depicted the mutual interactions of all the variables. Figure 1 (A) explains the interaction of inducing time and pH, where with increasing inducing pH level, RC yield achieved a quadratic gain. Therefore, a negative effect of interaction of these two variables was assumed, which could be seen from the negative sign of the coefficient of AB model term in the equation. There was a sharp convergence of the curve between pH 5.0 and 5.5, explaining inducing pH above certain limit would not contribute to increase RC yield further. Similarly, Figure 1 (B) explains the interaction of inducing time and inducing assistant amount. In this graph, RC yield increased with increase in both the variables till certain point, but there was a sharp convergence of the curve near the boundary, explaining that inducing time and inducing assistant amount above certain limit would not contribute to increase RC yield further.

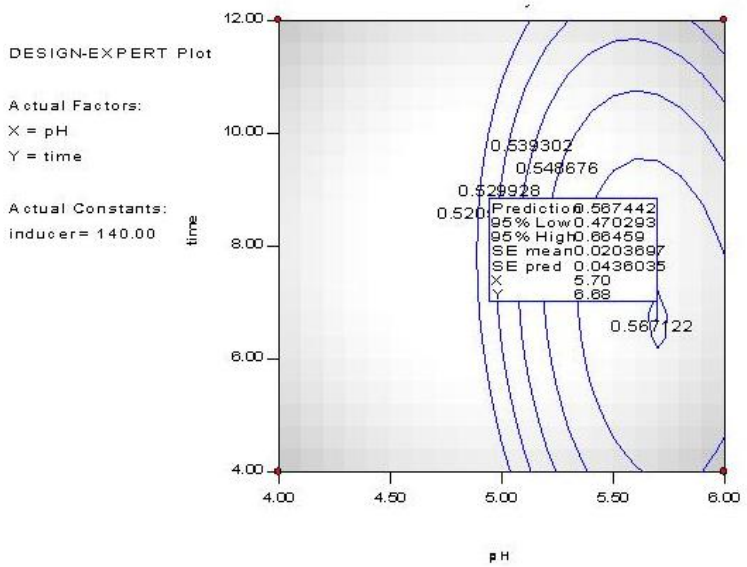


Fig. 2. Optimization of inducing condition for the yield of recombinant chum salmon cystatin.

Among the critical factors, inducing pH had the most significant impact on RC yield after the glucose in the medium was excluded. Because no carbon source and other nutrients could be applied in the medium, the appropriate amount of inducing assistant is helpful for the maintaining and expression of RC after induction by galactose. Besides, the inducing time showed similar impact like the previous study. RC yield reached the maximum up to certain inducing time. Therefore, the prediction was applied (Fig. 2.). In conclusion, the RSM was effectively used for the optimization of the process parameters for RC yield in *S. cerevisiae* YPH 499 and pH 5.70, inducing time of 6.68 h, inducing assistant of 5.6 g/L(YNB + amino acids + adenine) with galactose maintained at 2% were the desirable conditions for enhanced RC expression in *S. cerevisiae* YPH 499.

다. Effect of agitation speed

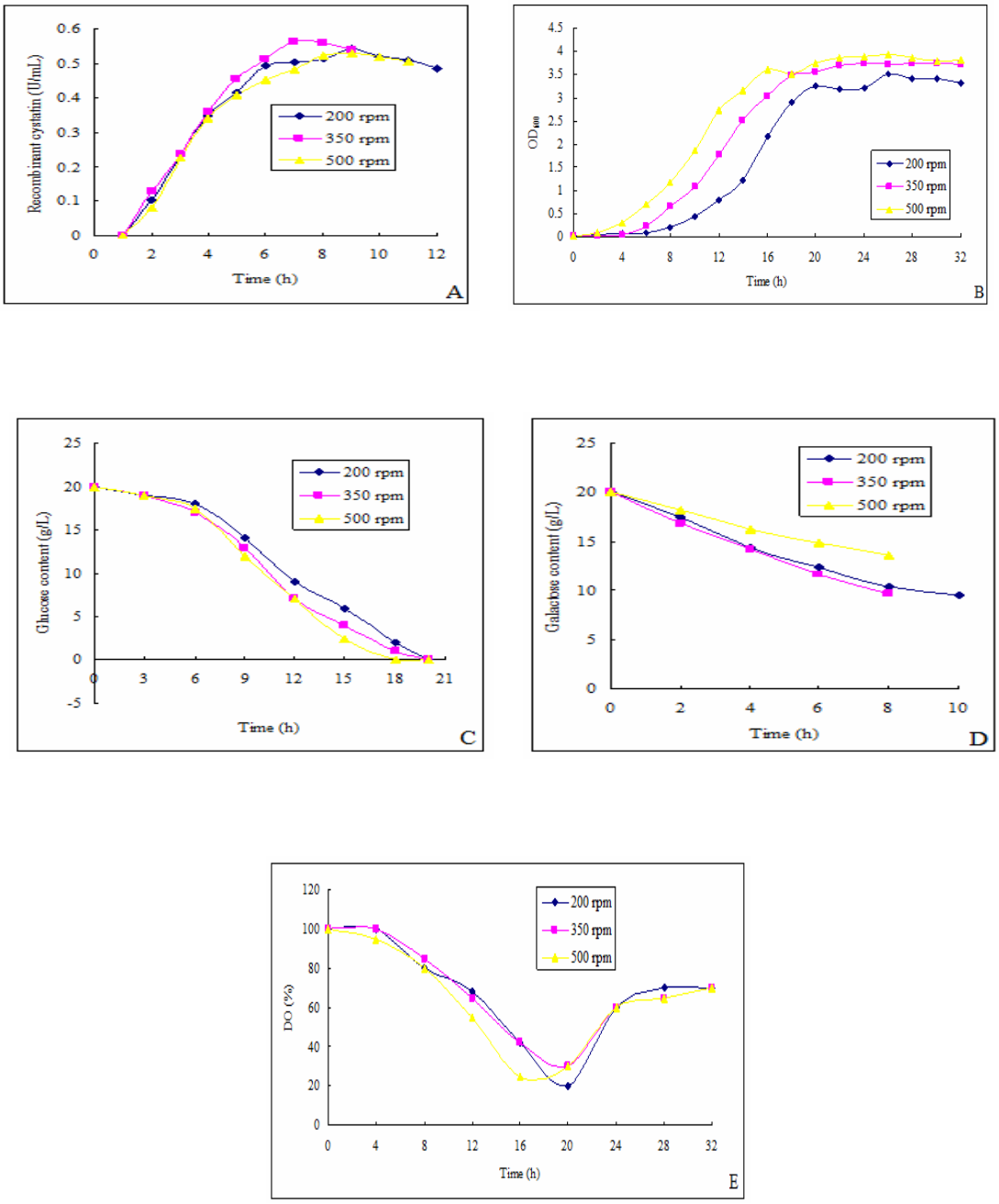
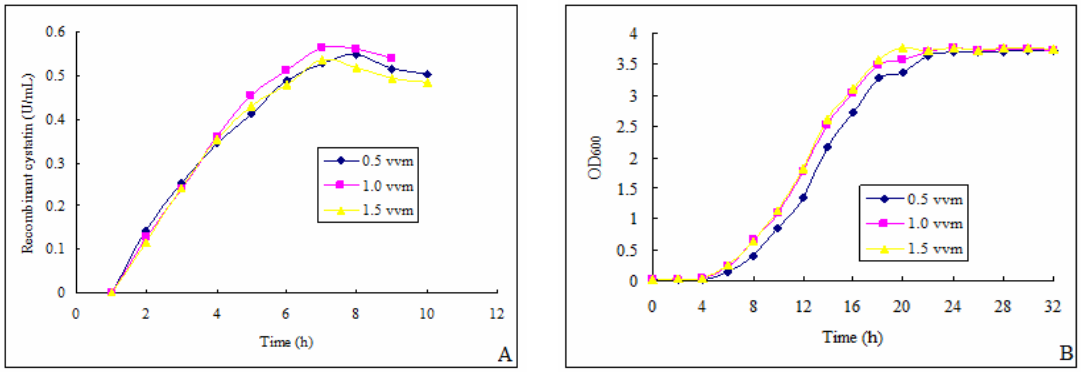


Fig.3. RC yield by *S. cerevisiae* YPH 499 at different agitation speed. (A) RC yield; (B) growth of *S. cerevisiae* (C) glucose content (D) galactose content (E) DO.

The fermentations were carried out at the constant temperature of 30℃ aeration rate of 1 vvm with different agitation speeds of 200, 350, and 500 rpm, respectively (Fig. 3. A-E). Increase in agitation is helpful to the growth rate and final cell density of *S. cerevisiae* (Fig. 3.B). Therefore, the consumption rate of glucose increased with the increase of agitation (Fig. 3. C). The changes were coincided with those in DO (Fig. 3. E). Fast growth of *S. cerevisiae* significantly decrease DO in the culture medium and reached the minimum when the glucose was excluded. Glucose was excluded after cultivation for 18 h when agitation was 500 rpm, which were 3 and 2 h earlier than those of 200 and 350 rpm. After glucose in the medium was excluded, galactose with certain ratio of inducing assistant (YNB: amino acids: adenine = 6.7:1.05:0.1) was added into fermentor to final concentration of 2% to induce the expression of RC. RC yield increased as the inducing time increased (Fig. 3. A), and maximum RC yield was reached at 9, 7, and 9 h under agitation of 200, 350, and 500 rpm, respectively. Maximal RC yield with agitation of 350 rpm was 0.56 U/mL and higher than other two. As the final cell density of 350 rpm was less than that of 500 rpm, agitation of 350 rpm showed the highest enhancing effect in RC yield. The highest consumption rate of galactose was obtained from that of 350 rpm and it was coincided with RC yield. Further increase of agitation was suggested to destroy the survival of yeast during induction by shear force (Shioya S. et al. 1999; Manolov RJ 1992).During induction, DO in the fermentor medium was recovered and kept on a stable level between 60% and 70%.

라. Effect of aeration rate



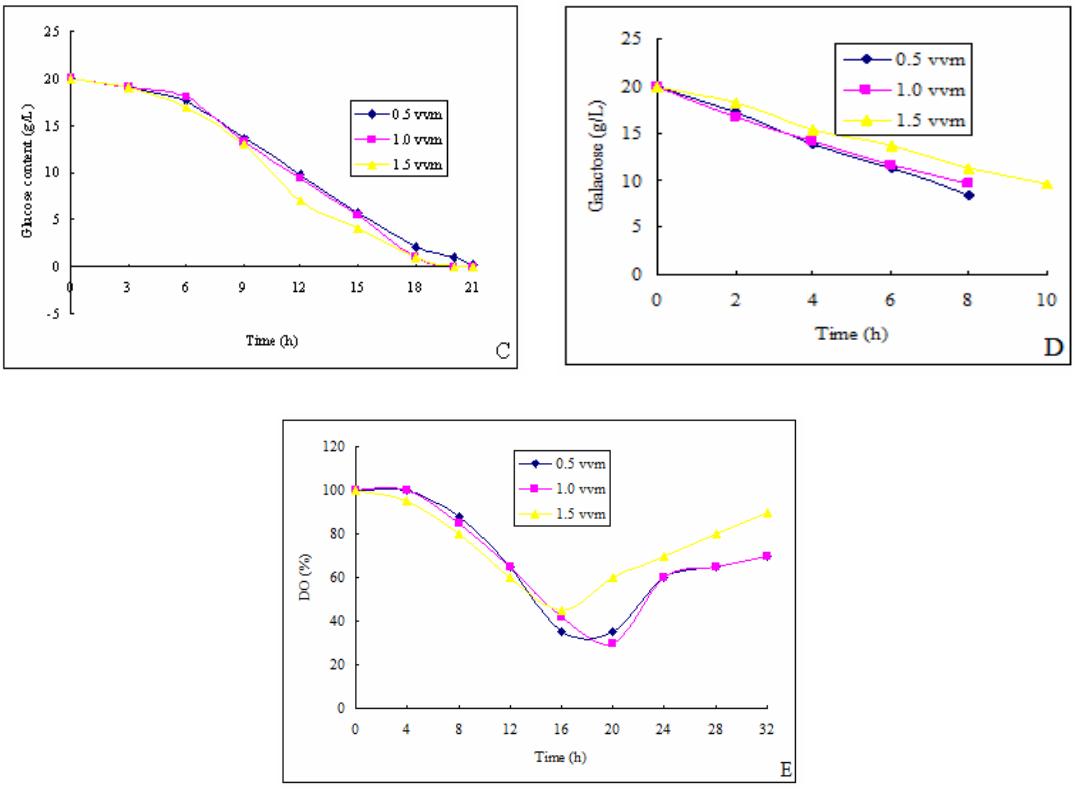


Fig.4. RC yield by *S. cerevisiae* YPH 499 at different aeration rate. (A) RC yield; (B) growth of *S. cerevisiae* (C) glucose content (D) galactose content (E) DO.

The fermentations were carried out at the constant temperature of 30 ℃ agitation of 350 rpm with different aeration rate of 0.5, 1.0, and 1.5 vvm, respectively (Fig. 4. A-E).

Increase of aeration is helpful to the growth rate but helpless to the final cell density of *S. cerevisiae* (Fig. 4. B). Consumption rate of glucose increased with the increase of aeration (Fig. 3. C). Glucoses were excluded after 21, 20, and 20 h under the aeration rates of 0.5, 1.0, and 1.5 vvm, respectively. DO greatly decreased during growth (Fig. 4. E). The decrease in DO with aeration rate of 1.5 vvm was the highest, and DO turned to increase after cultivation for 16 h. DO with aeration rate of 0.5 and 1.0 vvm decreased and reached the minimum when the glucose was excluded (Fig. 4. B, E). After glucose in the medium was excluded, galactose with certain ratio of inducing assistant was added as described above. The increase of aeration rate from 1.0 to 1.5 vvm decreased the galactose consumption rate and it was coincided with the changes in RC yield (Fig. 4. D, E). RC yield increased as the inducing time increased (Fig. 4 A), and maximum RC yield was reached at 8 h, 7 h, and 7 h under aeration of

0.5, 1.0, and 1.5 vvm, respectively. Maximal RC yield with aeration of 1.0 vvm was 0.56 U/mL and higher than other two. Because the final cell densities from three aeration rates were similar, and the difference of growth rate among three aeration rates were less than that among three agitation, the impact of aeration on the growth and RC yield is less than did agitation (Fig. 3. A, B; Fig. 4. A, B).

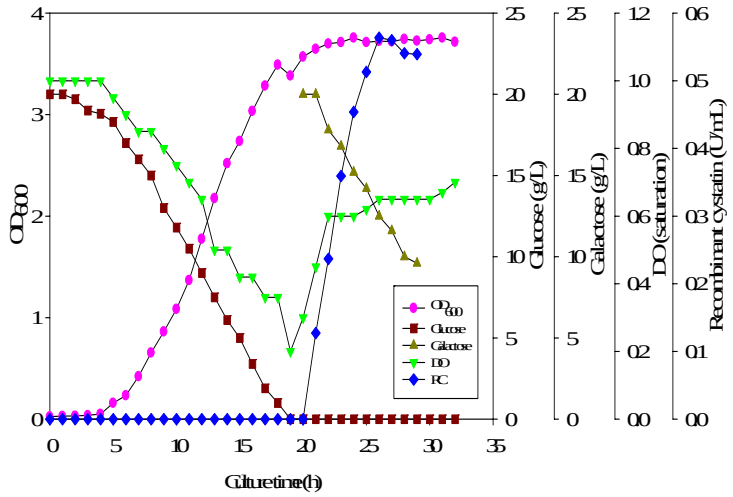


Fig. 5. RC yield by *S. cerevisiae* YPH 499 under optimal condition.

With temperature set at 30 ℃, pH of 5.67, agitation of 350 rpm and DO of 1.0 vvm, the changes of growth of *S. cerevisiae*, RC yield, glucose and galactose metabolism, and DO of the fermentation medium were plotted (Fig. 5.).

Table 3. Summary of the purification of RC from S. cerevisiae YPH 499 by alcohol

Alcohol concentration (v/v)	Total protein (mg)	Total activity (U)	Specific activity (U/mL)	Yield (%)	Purification (fold)
0	2.52	3.54	1.40	100	1
15%	0.34	0.32	0.94	9	0.67
30%	1.02	1.78	1.75	50	1.24
45%	1.16	2.89	2.49	82	1.78
60%	1.86	2.36	1.27	67	0.90
75%	2.11	2.18	1.037	62	0.74

The starting volume was 1000 mL cultivated broth. The inhibitory activity

was measured as decrease in papain activity. One unit of inhibitory activity was defined as the changes in absorbance at 440 nm per 30min.

The purification of RC was showed in Table 3. Alcohol was effective in protein separation, especially as the alcohol concentration increased. When alcohol was added to 30% and 45%, the purity of separated RC significantly increased. Further increase of alcohol was helpful for the precipitation of protein, but decreased the purity. Considering recovery and purification fold, 45% of alcohol addition was chosen as the optimal concentration.

In conclusion, temperature at 30 ℃, agitation at 350 rpm, pH of 5.67, DO of 1.0 vvm, and 7 h for induction were the optimal culture conditions for *S. cerevisiae* YPH 499 in fermentor using SC medium to produce RC. The maximum RC yield in fermentor, 0.56 U/mL, was about one and a half of that from baffled shake flask. Addition of cold alcohol to 45% (v/v) could help the purification of RC from *S. cerevisiae* YPH 499.

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제 5 장 목표 달성도 및 관련분야에의 기여도

제 1 절 연구목표 및 내용

1. 연구목표

O 어란 (연어, 청어, 명태, 물곰치, 빙어 알 등) 및 해조류 (녹조 및 홍조) 유래 단백분해효소저해제 정제 및 특성 연구, 대량 생산 기술 개발 O 단백분해효소 저해제를 이용한 수산식품의 품질 고급화를 도모함으로서 소비 확대, 수출증대 등 수산업 발전에 기여

1) 수산동물(어란)로부터 천연 단백분해효소 저해제의 검색, 분리 및 정제
2) 해조류로부터 천연 단백분해효소 저해제의 검색, 분리 및 정제
3) 수산동물 및 해조류 유래 천연 단백분해효소 저해제의 효과 및 저해기작 평가
4) 재조합 미생물의 개발 및 발효를 통한 수산물 유래 천연 단백분해효소 저해제의 생물공학적 대량생산공정 개발
5) 천연 효소저해제가 함유된 수산식품의 품질향상 평가

2. 연구내용

가. 천연 단백분해효소 저해제의 대량생산 및 수산식품에의 응용 기술 개발

1) 수산동물 (명태, 도치, 도루묵, 물곰치, 빙어, 연어) 알의 단백분해효소 저해제의 검색

- 단백분해효소 정제 (Ultrafiltration, Ammonium sulfate fractionation, Ion exchange chromatography, Gell permeation chromatography, affinity chromatography)
- 단백분해효소 저해활성 측정 (Papain activity 저해 활성 측정)
- 단백분해효소 저해제 순도 및 분자량 측정 (HPLC, SDS-electrophoresis)

2) 수산동물에 함유된 천연 단백분해효소저해제의 분리 및 정제

- 단백분해효소저해제 정제 및 특성실험
- 단백분해효소저해제의 효과 및 저해기작 분석
- 합성 및 천연 물곰치알 단백분해효소저해제의 효과 및 저해기작 실험
- Cathepsin 및 trypsin에 대한 물곰치알 저해제의 저해활성 비교분석

- 상업용 제품(Egg white 및 potato inhibitor)과의 비교 실험

3) 분리된 천연 효소저해제의 효과 및 저해기작 분석

- Ki and Vmax value 측정에 의한 저해기작 분석
- 온도 및 pH의 영향 분석
- 수분활성의 영향 분석

4) 효과가 우수한 수산물 유래 저해제의 아미노산배열 분석, 유전자 동정 및 PCR을 통한 유전자 복제

- 물곰치 저분자 단백분해효소저해제의 아미노산배열 분석, 유전자 동정 및 PCR을 통한 유전자 복제
- *E. coli* 균에 대한 cloning
- Cloned E. coli 균으로부터 재조합 단백분해효소저해제의 최적 배양조건 분석
- 재조합 단백분해효소저해제의 정제 및 활성 비교
- Calpain inhibitor의 저해활성 비교실험

5) 연어알 단백분해효소 저해제의 재조합 효모의 발효공정 및 효소저해제 생산의 최적화

- 효모에 발현된 연어알 단백분해효소 저해제의 배지 조성 및 생산의 최적화

6) 재조합 미생물의 발효를 통한 천연 효소저해제의 대량생산공정과 분리 정제기술의 확립

- 연어알 단배분해효소 저해제의 정제 방법 및 저해활성 연구
- 발효조를 이용한 대량생산
- 유기용매를 이용한 저해제의 대량정제

7) 재조합 효소저해제가 함유된 수산식품의 품질향상

- 단백분해효소저해제를 첨가한 surimi의 품질 특성 실험
- 화장품 관련 효소에 대한 저해활성 실험

나. 단백분해효소 저해제의 대량 생산 및 생산 공정의 최적화

1) 해조류(홍조 및 녹조)로부터 단백분해효소 저해제를 검색

- 해조류 2종(홍조인 방사무늬김, 녹조인 창자파래)으로부터 단백질분해효소 저해제 탐색
- 단백질분해효소 저해활성 측정

2) 해조류에 함유된 단백분해효소저해제의 분리 및 정제

- 홍조류(P. yezoensis and tenera) 및 녹조류(E. intestinalis)의 단백분해효소 저해제 효과의 비교 평가 및 P. yezoensis로부터의 단백질분해 효소저해제 분리공정효율 분석

3) 해조류 효소저해제의 효과 및 저해기작 평가

- *P. yezoensis*의 단백분해효소저해제의 특성을 온도 및 산도(pH)에 따라 beef plasma protein, egg white, potato powder 등 기존의 농·축산물 유래 저해제와 비교 분석

4) 물곰치 단백분해효소 저해제의 N-terminal 분석 및 합성

- 물곰치 알 단백분해효소저해제의 N-terminal을 근거로 peptide 합성

5) 연어알 단백분해효소저해제의 재조합 벡터 및 재조합 효모 개발

- Cystatin 발현 벡터 pYES2/NT_C (cystatin)를 효모(S. cerevisiae YPH499)에 형질전환
- 재조합 벡터를 제작해 효모에서 발현 실험

6) 물곰치알 재조합 벡터 및 재조합 효모 개발

- 재조합 벡터를 제작해 물곰치 유래 단백분해효소저해제의 형질이 전환된 효모개발

제 2 절 평가 착안점

구 분	평가의 착안점 및 척도	
	착 안 사 항	척 도 (점수)
1차년도 (2002년)	♣ 수산동물에 함유된 천연 단백분해효소 저해제의 검색 ♣ 해조류에 함유된 천연 단백분해효소 저해제의 검색	25
2차년도 (2003년)	♣ 수산동물 및 해조류에 함유된 천연 단백분해 효소 저해제의 분리 및 정제 ♣ 천연 효소저해제의 효과 및 저해기작 평가	25
3차년도 (2004년)	♣ 수산동물 유래 단백분해효소 저해제의 개발 ♣ 단백분해효소 저해제의 대량 생산 및 생산 공정의 최적화	25
4차년도 (2005년)	♣ 재조합 미생물의 발효를 통한 천연 효소저해제의 대량생산공정과 분리, 정제기술의 확립 ♣ 효소저해제가 함유된 수산식품의 품질 변화 평가	25
최종평가		100

제 3 절 목표 달성도 및 관련분야 기여도

1. 연구 개발 목표의 달성도

본 연구과제의 목표에 대한 달성도는 초과 달성되었다고 본다. 그 이유는 아래와 같은 실적이 생산되었기 때문이다.

○ 특허출원 : 국내 1건
○ 전문학회지 논문 게재 : 국내 1편, 국외 5편(SCI 4편, 1편은 인쇄 중)
○ 학회 및 세미나 발표 : 국내 5건, 국외 4건
○ 인력양성실적 : 석사 3명, 박사 1명

각 세부과제별로 수행한 연구 내용 및 결과는 아래와 같다.

가. 천연 단백분해효소 저해제의 대량생산 및 수산식품에의 응용기술 개발

○ 명란 (Alaska pollock egg)으로부터 단백분해효소저해제 정제 및 특성
○ 물곰치알 (Glassfish egg)로부터 단백분해효소저해제 정제 및 구조분석
○ 연어알 (salmon egg) 단백분해효소저해제의 정제 및 특성
○ 기타 어종(청어, 빙어) 단백분해효소저해제 정제 및 특성
○ 재조합 E. coli로부터 물곰치 단백분해효소저해제 정제
○ 재조합 효모의 연어알 단백분해효소저해제의 특성
○ 천연 효소저해제가 함유된 수산식품의 품질향상 연구

나. 단백분해효소 저해제의 대량 생산 및 생산 공정의 최적화

○ 단백질 함량이 높은 김 (홍조류)과 파래 (녹조류)에서 단백질 성분 추출 및 단백질분해효소저해제로서의 활성 연구
○ 해조 추출물의 단백분해효소 저해효과
○ 해조유래 저해제와 농, 축산 저해제와 와의 특성 비교
○ 연어알 단백분해효소 저해제의 효모 clonig

2. 관련분야 기여도

가. 학문적 활용에 대한 기여도

- 수산물 (어란 및 해조류)에 존재하는 천연 단백분해효소 저해제에 대한 정보 및 기초자료가 축적되어, 여러 가지 용도(학술, 양식, 유전공학)로의 활용

나. 경제·사회적 활용에 대한 기여도

- 유전자 조작 등에 의한 수산물 유래 단백질의 생물공학적 대량생산기술에 활용
-. 수산식품(맛살류)의 품질열화를 방지하여 저장기간을 증대하기 위한 효소저해제 이용 활용. 특히, 대표적인 수산식품인 맛살의 주요 품질열화 요인인 단백분해효소를 저해하여 수산식품 열화 억제 및 이로 인한 저장(유통)기간 연장에 활용

다. 교육·홍보 활용에 대한 기여도

- 수산물 유래 천연 효소저해제에 대한 생화학, 생리학적 기작 및 분리, 정제기술 연구에도 활용
- 유전자공학, 단백질공학, 발효공학, 분리/정제, 효소학, 식품공학 등 다양한 학문 기술 분야의 유기적인 연계활동을 촉진하여 우리나라 바이오산업의 활성화에도 활용
- 천연 단백분해효소 저해제의 상품화는 우선 참여기업에서 시행하도록 유도하며, 경쟁력 있는 새로운 바이오 벤처기업의 설립에 활용

제 6 장 연구개발결과의 활용계획

본 연구에서 얻어진 결과의 활용계획은 다음과 같다

1. 천연 단백분해효소 저해제의 상품화는 우선 참여기업에서 시행하도록 유도하며, 경쟁력 있는 새로운 바이오 벤처기업의 설립을 검토한다.

2. 맛살 제조, 유통 시 천연 효소 저해제를 첨가하여 품질열화를 방지 하여 획기적인 저장기간 연장을 꾀한다.

3. 수산발효식품의 품질을 유지하고 유통기간을 연장하여 세계화할 수 있는 전통제품으로 육성한다.

4. 수산식품 분야 뿐 아니라 농·축산식품 분야로 응용 범위를 확장한다.

5. 수산물 유래 천연 효소 저해제의 의약품으로서의 가능성을 검토한다.

6. 본 과제에서 확립된 효소저해제의 정제, 활성측정, cloning 등 기술적인 노하우는 관련 분야에 활용한다.

7. 본 과제에서 개발된 어란 유래의 단백분해효소저해제는 현재 Surimi 산업에서 많이 이용되는 난백단백질의 시장을 대체하도록 활용한다.

제 7 장 해외 과학 기술 정보

1. 미국

미국의 경우 곡류 유래 단백분해효소 저해제가 해충이나 곤충의 단백분해효소 활

성을 억제하여 피해를 줄이려는 방향으로 연구가 되어져 왔고 주로 대두, 감자, 수수 등의 곡류에서의 단백분해효소 저해제의 활성에 대한 연구 보고가 있다.

또한 미국은 현재 수산물은 명태 및 Pacific White fish를 원료로 한 맛살류의 시장이 증가하고 있다. 그러나 맛살류의 품질열화에 이용되는 효소저해제는 주로 식물성 단백분해효소 저해제를 이용하고 있으며, 어류 유래의 단백분해효소 저해제에 대한 연구는 아직까지 이루어지지 않고 있으나 단백분해효소에 대한 연구는 아주 활발한 편이다.

2. 일본

일본은 전통적으로 수산연제품인 맛살류(어묵)가 발달된 나라로 어묵에서의 단백분해효소의 활성을 억제하여 품질열화를 억제하려는 목적으로 많은 연구가 이루어지고 있다. 그러나 이들 저해제는 주로 육상동식물 유래의 것으로 gel 강도를 강화시키는 이점은 있지만 원하지 않는 색상이나 맛을 내는 부작용이 있으며, 또한 이들 저해제의 효과는 어종이나 맛살의 제조방법에 따라 많은 차이가 나고 있으므로 수산물에서의 단백분해효소저해제에 관한 연구가 활발히 이루어지고 있다. 일본의 경우 연어, 연어알 등에서 단백분해효소저해제를 분리·정제하였다는 연구가 보고되어 있으며, 주로 단백분해효소 분자의 진화과정을 밝히려는 목적으로 연구되고 있다. 그러나 수산물 유래 단백분해효소 저해제를 맛살류의 품질열화방지에 이용하려는 연구는 없다.

3. 동남아

동남아의 경우 수산연제품의 원료로 사용되는 어종들이 대부분 gel형성능이 떨어져 gel 형성능을 증가시키려는 연구가 이제 시작되고 있으며, 여러 어종들의 원료로써의 특성을 연구하고 있다. 그러므로 효소 저해제를 이용한 맛살류의 품질열화 연구는 이제 시작되고 있다. 지금까지 기초연구로써 잉어에서 단백분해효소저해제를 분리·정제하였다는 연구가 보고된 바 있다. 또한 잉어 유래의 cystatin저해제를 유전자 조작과 재조합 미생물을 이용하여 생산하려는 연구들이 보고되었다. 그러나 이러한 생물공학기술을 통한 저해제의 생산기술은 아직 개발단계에 있는 것으로 알려져 있다.

주 의

1. 이 보고서는 해양수산부에서 시행한 수산특정연구개발사업의 연구보고서입니다.

2. 이 보고서 내용을 발표할 때에는 반드시 해양수산부에서 시행한 수산특정연구개발사업의 연구결과임을 밝혀야 합니다.

3. 국가과학기술 기밀유지에 필요한 내용은 대외적으로 발표 또는 공개하여서는 안됩니다.