Peptidomics of Salmonella under Phagosome-Mimicking Culture Conditions

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Bioactive peptides are found in all branches of life

- **Peptidomics**: The study of native peptides (≤ 100 amino acids)
- Enormous diversity, especially given possible posttranslational modifications
- Usually discovered by working backwards from a function – low-throughput

**Can high-throughput comparative proteomics methodologies be used to identify bioactive peptides?**

**Function**

1. Iron Metabolism
2. Antimicrobial
3. Antifungal
4. Ca²⁺ Metabolism
5. Neuroactive
6. Protease Inhibition
7. Growth Regulation
8. Steroid Metabolism
9. Antiviral
10. Antitumoral
11. Hormone / Transmitter
12. Electrolyte Turnover
13. Fertility
14. Coagulation

_Curr Opin Biotech 2004 15:599–606_
Complementary *Salmonella* Investigations

- **Traditional bottom-up proteomics**
  - Proteins digested with trypsin
  - Poster MP12-254

- **Peptidomics** – The study of native peptides
  - No protein digestion
  - Natively occurring peptides must be isolated from intact proteins prior to analysis
A model system for peptidomic analysis: *Salmonella typhimurium*

**Human Pathogen**
- Causes ~7,000 cases of food poisoning annually in the US
- Used as a bioterror agent in Oregon, 1984

**Mouse Model of Typhoid Fever**
- Survives and thrives in macrophage phagocytic vesicles
- Uses a needle-like structure to inject toxins into host cytoplasm

*Salmonella* Infected Macrophage
N – Macrophage Nucleus
S – Phagocytosed *Salmonella*
Image courtesy of Dr. L. Shi, PNNL
Infection by *Salmonella typhimurium*

**Macrophages**
- Engulf (phagocytose) pathogens
- Block nutrients
- Digest pathogens

**S. typhimurium**
- Engulfed by macrophages
- Survive phagocytosis
- Evade immunodetection
- Parasitize macrophages

**Host-Pathogen “Combat”**
- Bioactive peptides are thought to have an important role
  - Macrophage antimicrobial peptides
  - *Salmonella* peptide toxins

*Salmonella*-Containing Vacuole
HM – Host Vacuolar Membrane
S – Phagocytosed *Salmonella*
Image courtesy of Dr. L. Shi, PNNL
Growth conditions for comparative analysis

- **Log**: Logarithmic phase cultures grown in a rich medium
  - Model of early-stage food contamination

- **Stat**: Early stationary phase cultures grown in a rich medium
  - Model of late-stage food contamination

- **MgM**: 4 hr shock designed to mimic early-stage phagosomal conditions
  - Acidic, low Mg$^{2+}$, and minimal nutrients

- **N-Salts**: 16 hr culture designed to mimic late-stage phagosomal conditions
  - N-Salts medium is similar to MgM medium

![Graph showing growth conditions](image)
Peptide Isolation Method 1 – Ultrafiltration

- Cleared *Salmonella* Lysate (Bead-Beating)
- Centrifugal Ultrafiltration (30,000 NMWF)

![Peptide separation diagram](image)

> Spin-filter clogged despite 20% ACN
> Poor results from MS analyses on filtrate
Peptide Isolation Method 2 – Isopropanol Extraction

- Cleared *Salmonella* lysate (bead-beating)
- Isopropanol protein precipitation $\rightarrow$ Centrifugation
- Protein pellet discarded and supernatant concentrated in speed-vac

$\rightarrow$ Isopropanol removed most proteins, as expected
$\rightarrow$ Good results from MS analyses on concentrated supernatant
Accurate Mass and Elution Time Tag Analysis

Peptides from Isopropanol Extraction → C18 SPE or SCX → nanoLC-Ion Trap MS² → Peak MS², Mass, NET

NET: Normalized Elution Time
Accurate Mass and Elution Time Tag Analysis

Peptides from Isopropanol Extraction → C18 SPE or SCX → nanoLC-Ion Trap MS² → Peak MS², Mass, NET → SEQUEST Spectrum Analysis (Normal & Scrambled) → Peptide IDs
Accurate Mass and Elution Time Tag Analysis

Peptides from Isopropanol Extraction → C18 SPE or SCX → nanoLC-Ion Trap MS² → Peak MS², Mass, NET

C18 SPE → nanoLC-FTICR MS

Peak Area, Mass, NET → Mass, NET Peak Matching → Abundance Information (Σ FTICR Peak Areas)

SEQUEST Spectrum Analysis (Normal & Scrambled) → Peptide IDs
Peptidome Data Analysis

Columns represent individual LC-MS analyses

Rows represent individual proteins (682 total)

Color scale depicts the Z-score – For each protein:
Step 1: $\Sigma$ FTICR peak areas of all peptides
Step 2: $Z$-scores calculated across rows

$$Z_i = \frac{x_i - \bar{X}}{\sigma} \quad \rightarrow \quad \text{A measure of relative abundance}$$
Peptidome and proteome co-cluster analysis

- Peptidomic and proteomic data was analyzed together.
- Z-scores were calculated separately.
- Weak correlation between peptidomic and proteomic abundances suggests targeted proteolysis.
Catabolism of ribosomal proteins

48 putative “growth proteins targeted for proteolysis”

37 are ribosomal proteins
Catabolism of ribosomal proteins

Proteome

Peptidome

Protein of Origin

12+ hr Starvation

↑ rRNA RNase

↑ rProtein Protease

Science 2001 293:705-8

J Biol Chem 2003 278:45041-4
Proteolysis of mistranslated proteins in stressed cells?

55 putative “virulence and/or stress response factors”

17 are known stress response factors, including rRNA RNase
Proteolysis of mistranslated proteins in stressed cells?

Starvation $\rightarrow$ ↓ Translational Fidelity $\rightarrow$ Protein Misfolding $\rightarrow$ Proteolysis

Conclusions

- Isopropanol extraction effectively isolated peptides
- Identification of 1000s of native Salmonella peptides
  - Discerning bioactive peptides remains an enormous challenge
- Putative identification of two peptidome/proteome clusters
  - “Growth proteins targeted for proteolysis”
  - “Virulence and/or stress response factors”

Future Directions

- Other techniques for isolating peptides
  - Acid precipitation → RP or SCX SPE
  - Restricted access media chromatography
  - Preparative electrophoresis
- Hypothesis driven experiments
  - lon, ppk, ppx, yej deletion mutants
- Peptides secreted by Salmonella
- Host-pathogen peptidomics
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