Proteases and protein instability – finally happening!

Elda Lerm
Technical Consultant
Anchor Yeast

INDUSTRY WORKSHOP
6 May 2015

Anchor Yeast
THE LEADING NEW WORLD WINE YEAST BRAND
AT FIRST I WAS LIKE

BUT THEN I WAS LIKE
Enological enzymes:
- used at several stages in processing: grape/must/wine
- complex cocktails of several enzymes
- varied concentration and activity; primary and secondary
- supplement/complement endogenous enzymes
  - effective
  - specific
  - convenient

Keep in mind:
Protein stability in wine

*Protein Stability* = ability of a protein to retain its structural conformation or its activity when subjected to physical/chemical manipulations
Protein sources in wine:
- synthesis during berry development 50%
- yeast protein synthesis during fermentation
- yeast autolysis

Protein levels in wine influenced by:
- more mature grapes
- extended lees contact
- skin contact
- warmer growing regions
- mechanical harvesting
- lower crop levels
**Protein haze** = visual defect / no flavour impact unacceptable to consumers

<table>
<thead>
<tr>
<th><strong>Problematic</strong></th>
<th><strong>Less problematic</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td>white wines</td>
<td>red wines*</td>
</tr>
<tr>
<td>lower phenol red varietals</td>
<td>wines fermented/stored in barrels</td>
</tr>
<tr>
<td>stainless steel fermented wines</td>
<td>*phenolic compounds react with proteins during fermentation</td>
</tr>
</tbody>
</table>

**Cause of protein haze** = grape pathogenesis-related (PR) proteins  
- infection/damage  
- 10 – 500 mg/L
Challenges of PR proteins:

Differences in:
- molecular weights
- unfolding temp’s
- structures
- stability
(greatly impacted by other grape components)
*Cause of protein haze* = grape pathogenesis-related (PR) proteins

1. thaumatin-like proteins (TLP)
2. chitinases
3. invertases
4. β-glucanases

↓

1. denaturation/unfold
2. aggregate
3. flocculate

↓

visible haziness

- unstable over time (storage conditions)
- (wine matrix composition)
- slow process; normal storage
Cause of protein haze = grape pathogenesis-related (PR) proteins

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↓

light-dispersing particles and visible haziness

reversible unfolding less likely to aggregate over short time periods
**Cause of protein haze** = grape pathogenesis-related (PR) proteins

1. thaumatin-like proteins (TLP)
2. chitinases
3. invertases
4. β-glucanases

\[ \text{unfold irreversibly} \quad \text{aggregate more aggressively} \]

\[ \downarrow \]

1. denaturation/unfold
2. aggregate
3. flocculate

\[ \downarrow \]

light-dispersing particles and visible haziness
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light-dispersing particles and visible haziness

native to non-native state:
  a) pH
  b) temp
  c) salt
  d) co-solutes
  e) preservatives
  f) intrinsic protein properties
**pH:**

low pH $\rightarrow$ smooth and homogenous haze  
high pH $\rightarrow$ coarse and flocculated haze

**Salt and co-solutes:**

- sulphate anions  $\rightarrow$ denaturation + aggregation  
- organic acids (-)  $\rightarrow$ prevents proteins (+) form sulphate interaction  
- polysaccharides  $\rightarrow$ inhibit aggregation  
- $\rightarrow$ stabilising effect on haze potential of proteins

**During fermentation:**

- $\uparrow$ soluble proteins  
- $\uparrow$ protein instability  
- $\Delta$ proportion of protein fractions

**After fermentation:**

- protein stability increases (stabilizing yeast polysaccharides)
1. Measure protein stability/haze potential

↓

2. Adjust protein content before bottling

↓

3. No haze formation during transport/storage

**INDUCE** haze

**MEASURE** haze

Protein stable – no treatment

Protein unstable – treatment

*Protein stability evaluation = just before bottling*

acidification/MLF/fortification/cold stab: precipitation of protein complexes
**INDUCE** haze

**MEASURE** haze

stable – no treatment

unstable – treatment

- **Heat test**

- **Bentotest**

- unreliable
- colour
- difficult
- observer variance

**Removal of proteins:**
- adsorption (Bentonite)
- precipitation

all proteins false unstable
**Bentonite**

- ✓ effective
- × efficiency
- × cost
- × impact on environment: waste disposal
- × impact on wine characteristics: quality
- × protein removal not selective
- × increased tank time

**Na**
- more reactive
- very fluffy lees
- higher % wine loss

**Ca**
- more compact lees
- lower % wine loss
- less effective, so use more

**Wine pH:**
- Bentonite: −
- Protein: +
**Amount of bentonite required:**

- **variety**
- **region**
- **vinification**

**Best time to fine and remove largest portion of proteins = juice**

- no loss of aromatic quality
- reduced amount of proteins for removal later
- less bentonite
- wine less altered by stabilisation process
Protease

- hydrolases
- cleavage of peptide bonds (links between amino acids) in proteins
- synthesis and degrading properties

= food / diary / detergent / leather industries etc.

Preparation, Properties and Possible Application of Coimmobilized Biocatalysts

W. Hartmeier

Summary

Recently, coimmobilizes have been developed which combine the biocatalytic properties of whole cells or parts of the cells and additional enzymes. The new method presented leads to very small immobilized particles with extraordinarily high specific activities and negligible diffusion barriers. Enzymatic properties
Endogenous proteases associated with grape and wine:

- Amino acids
- Autolysis
- Rot
**pepsin**

*chief digestive enzyme*

proteins → polypeptides

---

**trypsin**

*digestive enzyme*

---

**bromelain**

*anti-inflammatory*

---

**ficin**

*proteins → amino acids*

---

**papain**
Challenges of PR proteins:

- molecular weights
- unfolding temp’s
- structures
- stability

(greatly impacted by other grape components)
Right now?

- proteases not allowed for use in winemaking
- resolution for their approval in certain conditions
- “not approved for 2 more years”
- more applications for proteases will be investigated
# Materials Authorized for Treatment of Wine and Juice

<table>
<thead>
<tr>
<th>Materials and use</th>
<th>Reference or limitation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Protease (general)*</td>
<td><em>Aspergillus niger</em></td>
</tr>
<tr>
<td></td>
<td><em>Bacillus subtilisper</em></td>
</tr>
<tr>
<td></td>
<td><em>Bacillus licheniformis</em></td>
</tr>
<tr>
<td>Protease (Bromelain)*</td>
<td><em>Ananus comosus</em></td>
</tr>
<tr>
<td></td>
<td><em>Ananus bracteatus</em></td>
</tr>
<tr>
<td>Protease (Ficin)*</td>
<td><em>Ficus spp.</em></td>
</tr>
<tr>
<td>Protease (Papain)*</td>
<td>Carica papaya</td>
</tr>
<tr>
<td>Protease (Pepsin)*</td>
<td>Porcine or bovine stomachs</td>
</tr>
<tr>
<td>Protease (Trypsin)*</td>
<td>Porcine or bovine pancreas</td>
</tr>
</tbody>
</table>

* To reduce or to remove heat labile proteins
**Proctase**
- Aspergillopepsins I and II
- food grade
- well characterised
- active at wine pH and unfolding temp of PR proteins

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Reduction in protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>+ Proctase</td>
<td>20%</td>
</tr>
<tr>
<td>+ 1 min @ 75°C</td>
<td>40%</td>
</tr>
<tr>
<td>+ Proctase + 1 min @ 75°C</td>
<td>85-91%</td>
</tr>
</tbody>
</table>

→ NO BENTONITE NEEDED

- **heat treatment** 1 min @ 75°C
- unfolding
- protein most susceptible to attack
- aggregation
- Proctase 15 mg/L
- juice treatment = no negative sensory impact
Costing implications

Figure 3. Results of economic analysis of heating plus Proctase addition, compared with batch and in-line bentonite addition for Sauvignon Blanc, Chardonnay and Riesling juices (treatment cost in cents per L).
A promising enzyme for the stabilisation of white wines
New alternative to bentonite

Beyond bentonite
By Ella Robinson, Neil Scrimgeour, Matteo Marangon, Richard Muhlack, Paul Smith, Peter Godden and Dan Johnson
The Australian Wine Research Institute, PO Box 197, Glen Osmond, SA 5064.

Until now, bentonite treatment has been the winemaker’s best answer to troublesome haze-causing proteins. Breakthroughs in understanding the structure and properties of those proteins at the AWRI have led to the discovery of a potentially viable and practical alternative. Laboratory, pilot and industry-scale trials of proctase have now been successfully completed.

Proctase as a bentonite alternative – what’s the latest?
AWRI researchers have discovered information about the mechanisms of wine protein haze formation and have identified Proctase – with its ability to break down haze-forming grape proteins – as a potential alternative to bentonite.
working with OIV to gain approval for protease treated wines

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NEWS RELEASE

For immediate release:
Thursday, 11 December 2014

Way now clear for haze-preventing enzymes in Australian winemaking

Proctase + flash past. = available for 2015 vintage

APPROVED: Food Standards Australian New Zealand (FSANZ)
Use in AUS winemaking: wines sold domestically and exported to NZ
Current Winetech funded research
IWBT

Identification and Partial Characterization of Extracellular Aspartic Protease Genes from Metschnikowia pulcherrima IWBT Y1123 and Candida apicola IWBT Y1384

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produced and secreted by the yeast
Current Winetech funded research

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acid protease
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non-Saccharomyces
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- enzyme is secreted in presence of grape juice proteins

*Still to determine:*
- optimum pH and temp. for enzyme activity?
- activity of enzyme in wine?
- ability to hydrolyze wine proteins and significantly reduce haze formation?
description of the principal products used to make wine

conditions, instructions and limits for their use

337 pages...0 mentions

708 pages...1 mention
### 2015 Annual Work Programme

*In accordance with the axes of the OIV 2015-2019 Strategic Plan*

<table>
<thead>
<tr>
<th>SP Reference</th>
<th>Start Date</th>
<th>Actions</th>
<th>Expected in 2015</th>
<th>Lead (in bold) and other structures involved</th>
</tr>
</thead>
<tbody>
<tr>
<td>2 cl</td>
<td>2014</td>
<td>Drafting of oenological practices; Use of proteases</td>
<td>Presentation of the opinion and draft resolution</td>
<td>CII, CIV</td>
</tr>
<tr>
<td>4 al</td>
<td>2014</td>
<td>Evaluation and opinion on the new additives or processing aids proposed as new practices</td>
<td>Opinion given and published on: - Polydimethylsiloxane - Agar-agar - Potassium carbonate - Protease - Polyamines</td>
<td>CIV</td>
</tr>
</tbody>
</table>

*Further information...*

*Consideration...*

*Opinion...*

*Progression...*
In the future?

- inclusion of protease in the International Oenological Codex of the OIV

- yeast producing extracellular proteases during fermentation

- other proteases under investigation:
  - Bcap8 (B. cinerea)
  - Antarctic fungi

- Carrageenan (red seaweeds)
Other issues to address with protease applications:
**Alternative options to proteases for treatment of protein stability**

Any alternative to bentonite

→ cheaper/more cost effective
→ better settling with less waste
→ more efficient
→ no flavour taint

**Mannoproteins**

- compete for non-protein compounds that contribute to instability
- positive impact on wine colour

**Molecular imprinted polymers**

- “designed” to selectively remove proteins
Proteases and protein instability – finally happening...
but not just yet!

Elda Lerm
Technical Consultant
Anchor Yeast

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