IMMOBILIZATION OF THE ENZYME POLYPHENOL OXIDASE ON DENDRISPHERES

IN PARTIAL FULFILMENT OF THE DEGREE

MAGISTER SCIENTIAE

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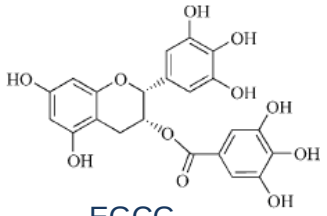
26112664

April 2011

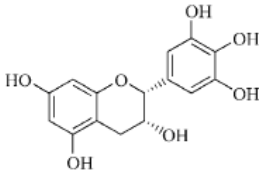
CSIR

our future through science

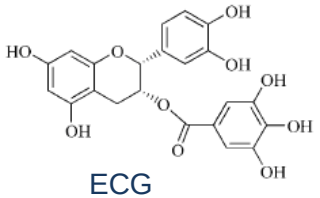
TEA (*Camellia sinensis* plant)



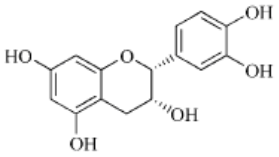
EGCG



EGC



ECG



EC

Second most consumed beverage in the world

Grouped into:

- •Green tea — Non-fermented
- •Oolong tea — Partially fermented
- •Black tea — Fermented

Catechins:

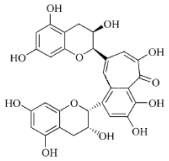
- •Major component present in green tea leaves
- •(-)-Epigallocatechin gallate (EGCG), (-)-epigallocatechin (EGC), (-)-epicatechin gallate (ECG) and (-)-epicatechin (EC)

During fermentation :

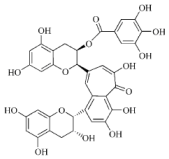
- •Catechins undergo polyphenol oxidase-dependent oxidative polymerization
- •Leading to the formation of theaflavins, thearubigins and other oligomers

Leung L. K. et al. (2001) *American Society for Nutritional Sciences* **131**; 2248-2251 and Pan M. et al. (2000). *Biochemical Pharmacology* **59**; 357-367

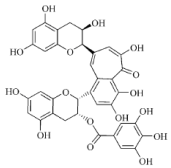
THEAFLAVINS



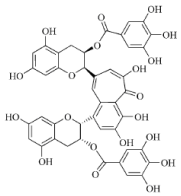
Theaflavin



Theaflavin-3-gallate



Theaflavin-3'-gallate

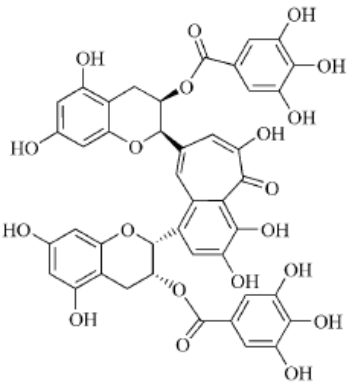


Theaflavin digallate

- Contain a system of benzotropolone rings with di- or trihydroxy substitutions
- Theaflavin, theaflavin-3-gallate, theaflavin-3'-gallate and theaflavin-3,3'-digallate
- Principle pigment present in black tea
- Contribute to the briskness and brightness of black tea
- Antioxidant-, anticancer-, antimutagenic-, acticlastogenic-, antiviral- and antibacterial activity

Owuor P. O. and McDowell I. (1994) *Food Chemistry* **51**; 251-254; Leung L. K. et al. (2001) *American Society for Nutritional Sciences* **131**; 2248-2251; Leone M. et al. (2003) *Cancer Research* **63**; 8118-8121; Halder B. et al. (2005) *Food and Chemical Toxicology* **43**; 591-597; Nashimoto K. and Tashiro Y., Marsushita Seiko Co., Ltd., Mitsui Norin Co., Ltd. (1999) Gargling cup, antiviral mask, antiviral filter, antifungal, antibacterial, and antiviral filter air cleaner and air-cleaner humidifier, 5,888,527 and http://www.healthabs.com/wp-content/uploads/2010/12/black-tea-111.jpg

THEAFLAVIN-3,3’-DIGALLATE



Theaflavin digallate

- Antiproliferative activity on tumour cells
- Potential as a chemopreventive agent
- Antiviral activity by inhibition of virally encoded 3C-like protease

(Presumed essential for viral replication of the causative agent of SARS)

- In general could prove as a therapeutically effective anti-inflammatory treatment
- Antioxidant activity strongest amongst theaflavins and catechins

(Analysis TBARS and conjugated dienes produced during LDL oxidation)

Liang Y. et al. (1999) *Carcinogenesis* **20**(4); 733-736; Lee H., Ho C. and Lin J. (2004) *Carcinogenesis* **25**(7); 1109-1118; Chen C. et al. (2005) *eCAM* **2**(2); 209-215; Lin Y. et al. (1999) *European Journal of Pharmacology* **367**; 379-388 and Leung L. K. et al. (2001) *American Society for Nutritional Sciences* **131**

THEAFLAVIN SYNTHESIS

- Research hampered by low abundance and challenging purification procedure
- Resulting in a focus on the use of mixtures of theaflavins
- Methods to acquire theaflavins:

1. Extraction from black tea
    - During fermentation of the15-30% of catechins present green tea only 0.4-1.85% theaflavins formed
    - Industrial unfeasibility and high cost
2. Oxidation of catechins by polyphenol oxidase in a fermentation system
3. *In vitro* modelling of black tea fermentation
    - Utilizing chemical oxidation and /or enzymatic oxidation (polyphenol oxidase)

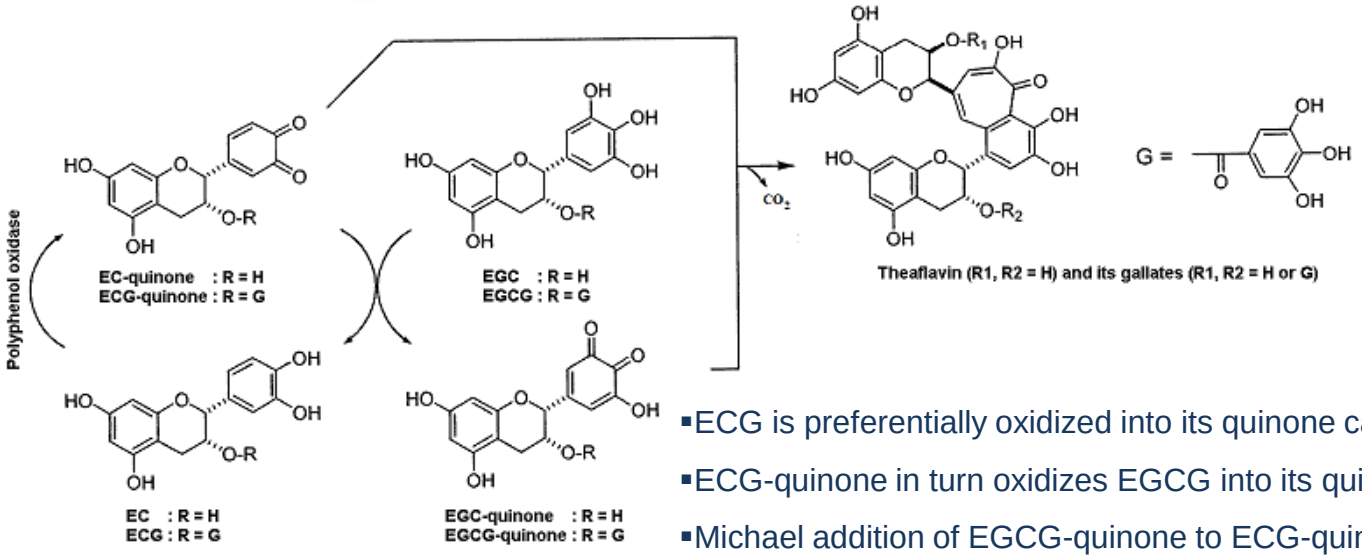
Sang S. et al. (2004). *Bioorganic & Medicinal Chemistry* **12**; 459-467; Sharma K., Bari S. S. and Singh H. P. (2009). *Journal of Molecular Catalysis B: Enzymatic* **56**; 253-258 and Wang C. and Li Y. (2006). *African Journal of Biothechnology.* **5**; 213-218

POLYPHENOL OXIDASE

- Found in plants (most fruit and vegetables) fungi, amphibian, arthropods and mammals
- Biogenesis of melanin, enzymatic browning fruit and vegetables
- Pear and loquat homogenates:
  - Much larger theaflavin synthesis capacity than fresh tea leaves
  - Highest among 62 plants belonging to 49 families
- Type-3 copper enzyme with a dinuclear copper centre
- Mixed function oxidase:
  - Monophenolase activity (*ortho*-hydroxylation of monophenols)
  - Diphenolase activity (oxidation of *o*-diphenols to *o*-diquinones)

Gerdemann C. Eicken C. and Krebs B. (2002).*Acc. Chem. Res.* **35**; 183-191; Matoba Y. et al. (2006). *The Journal of Biological Chemistry* **281**(13); 8981-8990; Granata A. et al. (2006). *em. Eur. J.* **12**; 2504-2514; Miranda M. et al. (1985). *Biochimica et Biophysica Acta* **841**; 159-165; Martinez M. V. and Whitaker J. R. (1995). *Trends in Food Science & Technology* **6**; 195-200; Riley P. A. (1997) Melanin. *Int. J. Biochem. Cell Biol.* **29**(11); 1235-1239; Bachem C. W. B. et al. (1994). *Bio/Technology* **12**;1101-1105 and Tanaka T. et al. (2002). *J. Agric. Food Chem.* **50**; 2142-2148

DIPHENOLASE ACTIVITY OF POLYPHENOL OXIDASE



Compounds	Parent Flavonols
Theaflavin	EC + EGC
Theaflavin-3-gallate	EC + EGCG
Theaflavin-3'-gallate	ECG + EGC
Theaflavin-3,3'-digallate	ECG +EGCG

- ECG is preferentially oxidized into its quinone catalyzed by polyphenol oxidase
- ECG-quinone in turn oxidizes EGCG into its quinone
- Michael addition of EGCG-quinone to ECG-quinone produces a 3-membered ring intermediate
- The intermediate is oxidized and decarboxylated to form a troponoid skeleton, theaflavin-3,3'-digallate
- Reduction of dimer quinones produces theasinensins
- Complexity is increased by destruction of theaflavins by their conversion into thearubigins through the catechin quinones

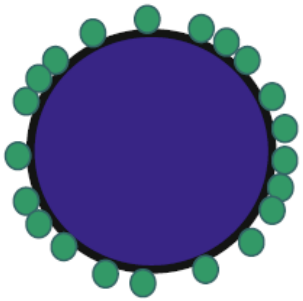
Jhoo J. et al. (2004). *Journal of Food Lipids* **11**; 89-103; Tanaka T. and Kouno I. (2003). *Food Sci. Technol. Res.* **9**(2); 128-133 and Hodges G. H., Lipton, division of Conopco, Inc. (2008) Process for producing theaflavins, US 2008/0131558 A1

ENZYME IMMOBILIZATION

- Overcomes factors hampering the application of enzymes for a given reaction:
  - Lack of long-term stability under process conditions
- More rigid enzyme structure decreases its sensitivity to thermal deactivation
  - Difficulties in recycling and recovering of the enzyme
- Easy separation from the reaction mixture and continuous or repeated use
- Further advantages:
  - Enhanced enzyme activity
  - Modification of substrate selectivity and enantioselectivity
  - Multi-enzyme reactions
  - Possibility to modulate enzyme catalytic properties

Brady D. and Jordaan J. (2009) *Biotechnol Lett* **31**; 1639-1650 and Petersen M. and Kiener A. (1999) *Green Chemistry* **1**; 99-106

ENZYME IMMOBILIZATION TECHNIQUES



Solid support

Noncovalent adsorption or deposition:

- Adsorption
  - •Aqueous media (leaching)
  - •Important parameters include the enzyme concentration, nature of the carrier surface (porosity and pore size) and size of the protein
- Depostion

•Van der Waals interactions

(large lipophilic surface areas for efficient immobilization)

•Hydrogen bonds

(dependent on enzymes hydrophilicity due to hydrophilic amino acid residues as well as glycosylation of the enzyme)

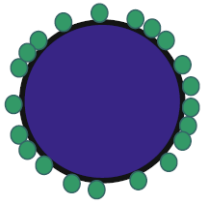
•Ionic bonds

(strongly dependent on pH and salt concentration)

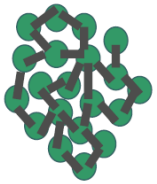
•Hydrophobic interactions

Bornscheuer U.T. (2003). *Angew. Chem. Int. Ed.* **42**; 3336-333; Hanefeld U., Gardossi L. and Magner E. (2009). *Chem. Soc. Rev.* **38**; 453-468 and Villeneue P. et al. (2000). *Journal of Molecular Catalysis B: Enzymatic* **9**; 113-148

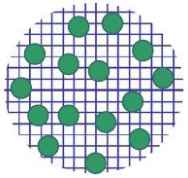
ENZYME IMMOBILIZATION TECHNIQUES



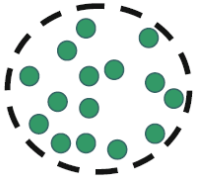
Solid support



Self immobilization



Entrapment



Encapsulation

Covalent attachment

- •Tightly fixed on solid support minimizes leaching in an aqueous medium
- •Multiple bonds prevent enzyme unfolding and denaturation
- •Disadvantaged by chemical modification of the enzyme
- •Can be attached via a spacer arm to the support
- •Could provide washing and reusing possibilities without loss of activity

Cross-linking of an enzyme

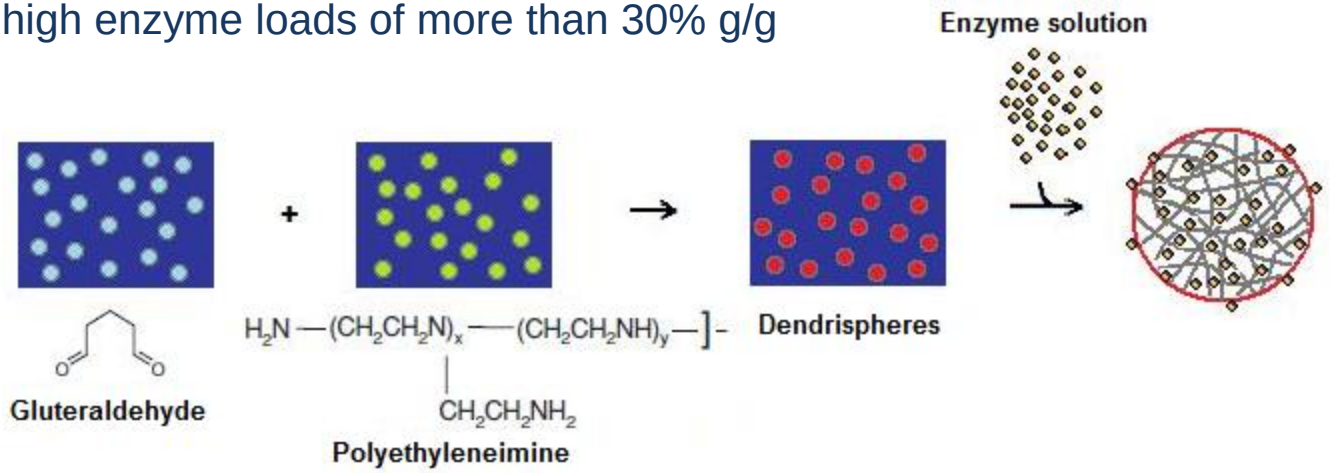
Entrapment in a polymeric gel, membrane or capsule

- ▪Entrapment
  - •Immobilize large amounts (great variation) of enzyme without chemical alteration
  - •Common approach is a hydrophilic catalyst in a polar interior of an amphililic support
- ▪Encapsulation
  - •Artificial cells delimited by a membrane
  - •Limited by diffusion rate of substrate and product across the membrane

Bornscheuer U.T. (2003). *Angew. Chem. Int. Ed.* **42**; 3336-333; Hanefeld U., Gardossi L. and Magner E. (2009). *Chem. Soc. Rev.* **38**; 453-468 and Villeneue P. et al. (2000). *Journal of Molecular Catalysis B: Enzymatic* **9**; 113-148

DENDRISPHERES

- Emulsion-derived particles
  - Loosely linked polymer network
  - Interstitial openings around and adjacent to the strands
  - Polymer is polyethyleneimine (PEI) and the cross-linking agent gluteraldehyde
- Covalent (interaction of the epoxide or aldehyde with amine groups of the protein)
- Hydrophobic or affinity bonding can be achieved through polymer network modification
- Extremely high enzyme loads of more than 30% g/g



Jordaan J., Simpson C. and Gardiner NS (2009a) Emulsion-derived particles. Patent WO2009/057049

AIMS

1. Purification of polyphenol oxidase from pear.

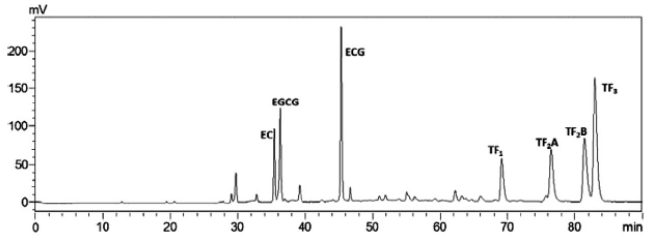
•Identification of the pear variety with the highest theaflavin synthesis capacity in green tea based on the flavognost method

•Purification by a Sepharose 4B-L-tyrosine –*p*-amino benzoic acid affinity column

•Analysis via enzyme activity, protein concentration, SDS-PAGE, zymogram and purification table

2. Measurement of Theaflavins by HPLC/UV

•Standards: (-)-Epigallocatechin gallate (EGCG), (-)-epigallocatechin (EGC), (-)- epicatechin gallate (ECG), (-)-epicatechin (EC), Theaflavin (TF_1), theaflavin-3-gallate (TF_2A), theaflavin-3'-gallate (TF_2B) and theaflavin-3,3'-digallate (TF_3)



Arslan O. et al. (2004). *Food Chemistry* **88**;479-484 and Xu Y, Jin Y, Wu Y. and Tu Y. (2010) *Journal of Liquid Chromatography & Related Technologies* **33**; 1791-1801

AIMS

3. Comparative assessment polyphenol oxidase immobilized on Dendrispheres with immobilization on Eupergit C, Eupergit C 250L and DEAE Sephadex A-25 based on enzyme loading capacity, activity, stability and recyclability

•Dendrisphere synthesis and determination of particle size and dry weight

•Immobilization of polyphenol oxidase on the different supports (preliminary assessment with BSA)

•SEM of Dendrispheres and polyphenol oxidase immobilized Dendrispheres

•Loading capacity (% gram enzyme/ gram support), stability (residual activity of the free and immobilized enzymes during storage), reusability (number of batches per turnover without loss of activity)

4. Optimization of conditions for theaflavin formation for both free and Dendrispheres immobilized polyphenol oxidase

•Temperature- and pH- activity profiling

•Control mushroom tyrosinase (Sigma-Aldrich)

Jordaan J., Simpson C. and Gardiner NS (2009a) Emulsion-derived particles. Patent WO2009/057049

HYPOTHESIS

$H1_0$: There will be no statistical significant difference in the yield of theaflavins/Unit enzyme activity between the free and Dendrispheres immobilized Polyphenol oxidase at the 95% level of confidence.

$H1_1$: There will be a statistical significant difference in the yield of theaflavins/Unit enzyme activity between the free and Dendrispheres immobilized Polyphenol oxidase at the 95% level of confidence.

EXPECTED RESULTS

Pear homogenate theaflavin yield of 55.6 mol% from 16.3 mmol epigallocatechin

Single band on SDS-PAGE and a molecular weight of ≈ 65kDa

Dendrisphere particle size of ≈ 3nm and XXX dry weight

Loading capacity

- •Polyphenol oxidase loading capacity of more than 30% g/g on Dendrispheres
- •Much higher than the 1.18% g/g reported for tea leaf polyphenol oxidase immobilized on activated cellulose matrix

Reusability

- •Bioconversion of catechins into theaflavins without loss of activity for first ≥ 14 number of batches

Stability

- •After 30 days of storage at 4°C to retain ≥ 90% enzyme activity

Tanaka T. et al. (2002). *J. Agric. Food Chem.* **50**; 2142-2148; Sharma K., Bari S. S. and Singh H. P. (2009). *Journal of Molecular Catalysis B: Enzymatic* **56**; 253-258; Arslan O. et al. (2004). *Food Chemistry* **88**;479-484; Jordaan J., Simpson C. and Gardiner NS (2009a) Emulsion-derived particles. Patent WO2009/057049 and Brady D. and Jordaan J. (2009) *Biotechnol Lett* **31**; 1639-1650

EXPECTED RESULTS

Higher enzyme activity of the immobilized polyphenol oxidase than the free enzyme at extremes of temperature and pH

Bioconversion efficiency of catechins into theaflavins

- > 0.4-1.8% theaflavin formation during black tea manufacturing
- ≥ 85% theaflavin formation by polyphenol oxidase immobilized on activated cellulose matrix

Brady D. and Jordaan J. (2009) *Biotechnol Lett* **31**; 1639-1650 and ; Sharma K., Bari S. S. and Singh H. P. (2009). *Journal of Molecular Catalysis B: Enzymatic* **56**; 253-258

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QUESTIONS