Final Research Progress Report

# Development of a Rapid Test Kit for Yolk Contamination in Egg White Using Thin Film Chemistry (Reagent Strip Chemistry)

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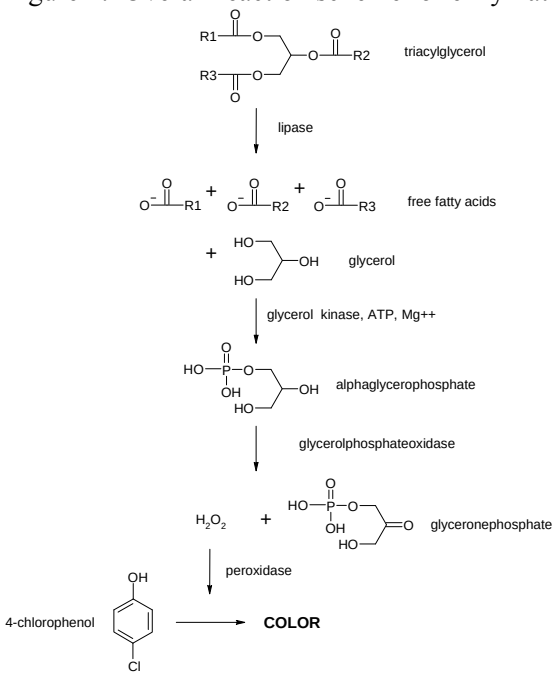
## [1] Technical

The overall objective was to develop an enzyme-based test strip which could be used to quantify triacylglycerols in egg yolk contaminated egg whites as a method of measuring the level of contamination. The overall reaction scheme that we studied is shown below. The extent of the 4-chlorophenol oxidation would be determined visually with comparison to reference.

Figure 1. Overall reaction scheme for enzymatic determination of triacylglycerols



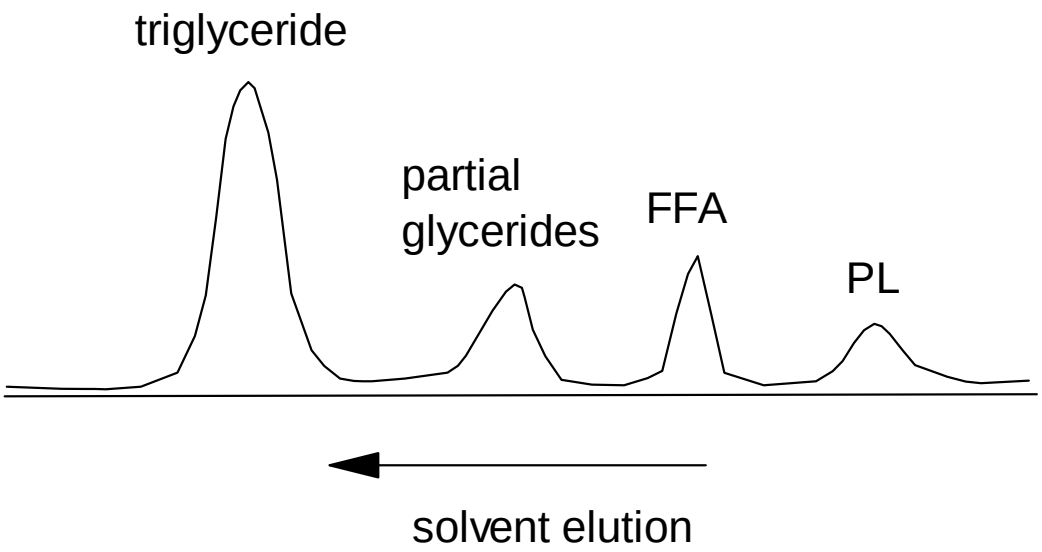
Lipases

Experiments were conducted to determine the optimal lipase enzyme source and hydrolysis conditions. The enzyme sources are shown in the Table below. All were obtained from Sigma except for *Rhizopus* (Biochemika) and *Pseudomonas* (Fluka). We originally began experiments using synthetic triglyceride (triolein) but realized quickly that for optimal applicability to egg lipids, using egg lipid made much more sense due to enzyme fatty acid specificity effects. All of the data presented here originate from egg lipid.

Fresh eggs were obtained from a local supermarket. Lipid was extracted from egg yolk by using the procedure described by Bligh and Dyer (1959). Solvent was removed using a Rotory evaporator at 50 °C (Buchi R3000).

VII Candida rugosa
Thermomyces lanuginosus
Rhizomucor miehei in Asperguillus oryzae
Mucor meihei
Mucor javanicus
Rhizopus arrhizus
Chromobacterium viscosum
Pseudomonas cepacia

Separation of intact triglyceride from free fatty acids and partial glycerides (mono- and diglycerides) was examined by using thin layer chromatography combined with flame ionization detection (Iatroscan Mark IV, Iatron Lans, Tokyo Japan) with S-III chromarods. This type of thin layer chromatography allows detection of microgram levels of lipid by combining separation on silica gel impregnated quartz rods with a flame ionization detector. A typical chromatogram obtained from an egg yolk sample that was partially hydrolysed by lipase is shown below.

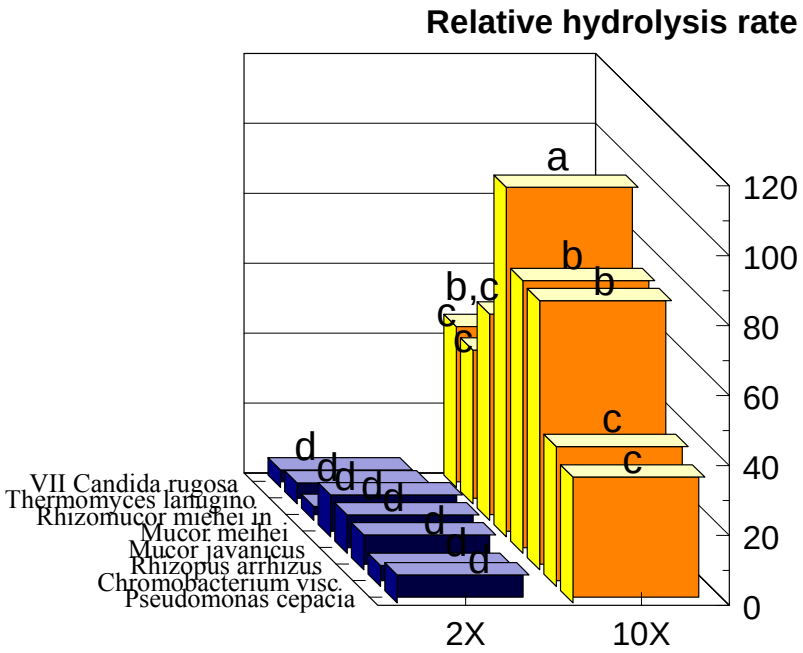


Initial experiments were designed to determine the rate of lipase activity in egg white that has been artificially contaminated with 0.05% egg yolk. Assuming a 26% lipid in yolk, this would result in a lipid concentration of 0.013g lipid in 100g contaminated white. So the amount of lipid that requires hydrolysis is 13 mgs in 100g, 0.05% yolk-contaminated white. About three-fifths of the lipid is triglyceride, with a third phospholipids and 5% sterols. So 13 mgs of lipid would represent about 8 mgs of triglyceride. So in a 100g batch of contaminated egg yolk, the amount of lipid that would be available for hydrolysis to glycerol is between 2 to 32mg of triglyceride, for contamination levels between 0.01% to 0.2%. The amounts of enzyme required for complete hydrolysis of this amount of triglyceride is less than 1 unit (meq hydrolysed per hour), but the rate of hydrolysis can't be so slow to require 1 hour. To ensure that the rate of hydrolysis was adequate for an analytical strip, 2 and 10 fold excess of enzyme were examined.

Egg lipid (30mg) was emulsified with 1g phosphate buffered water (10mM, pH 7.6) containing 0.2g Tween 20 (Sigma) and added enzyme by using two syringes connected with a thin tube. The lipid-water solution was mixed back and forth 10 times. Emulsions did not separate over the incubation time (30 minutes). The average triglyceride weight was assumed to be 885g/mole (i.e., triolein), and there are 3 equivalents of fatty acid per mole, so the actual units of enzyme used were 200 and 2000, representing 2 and 10 fold excess enzyme activity. Most of the enzyme activities are reported for 60 minute incubation at 37 ˚C at pH 7.2 to 7.7. To ensure that the pH was not outside the range for optimal activity, the water was buffered using a dilute 10mM phosphate. The enzyme was added to the aqueous component and allowed to rehydrate for 5 minutes before emulsification.

Thin layer chromatography was conducted using Baker Si250PA 20cmx20cm plates using 90:10:1 (hexane, diethyl ether, acetic acid, v/v/v). Spots were visualized by using UV light after spraying with 2', 7'-dichlorofluorescein. A Spectroline CC-80 light box was fitted with a Sony digital camera and was used with Uniscan integration software running on an IBM PC for area quantitation of the spots. Hydrolysis rate was determined by comparing the initial and final amount of triglyceride and was converted to mg/30 minutes. Data were normalized for the highest activity.

Temperature for lipase hydrolysis is usually reported to be around 35-40 ˚C. There would be advantages of a reaction that could be evaluated at refrigeration temperatures, so reactions were evaluated at 37 ˚C and 5 ˚C. Initial studies at 5 ˚C showed that the hydrolysis rates were very poor compared to 37 ˚C (rates about 10% of the higher temperature) and would result in unacceptably long reaction times. The results for 40 ˚C are shown below. The *Mucor meihei* enzyme had a significantly higher rate at 10X enzyme and lower enzyme levels resulted in poor hydrolysis for all enzymes. We decided to use the *meihei* enzyme for further development.




Glycerol Kinase

Glycerol kinases were obtained from 4 sources (below). Amano 2 was obtained from Amano, *E. coli* from Fluka and the others from Sigma. α-glycerophosphate was separated from glycerol by derivitization using hexamethyldisilazine and trimethylchlorosilane (HMDS/TMCS) in pyridine (Nishihara et al., 1999) with analysis using a Simadzu QP5050 GCMS fitted with a 60m x .25mm i.d., 0.25µm film DB-5 column (J&W, Folsom CA). Many other analysis methods have been reported (Westergaard et al., 1998; Sadava and Moore, 1987), including coupled reactions with pyruvate kinase and lactate dehydrogenase with reaction followed by NADH oxidation, but the GC appeared to be the most specific and sensitive.

*Cellulomonas*
Amano 2
*Bacillus stearothermophilus*
*Escherechia coli*

Some of the enzymes have optimal activities around pH 8.9-9.8 (Worthington Biochem, *Cellulomonas*), which is outside the optimum enzyme activity range for the lipases studied. Because it would not be possible to have one pH where all enzymes are at their optimum, we chose to stay just on the basic side of neutrality To determine glycerol kinase activity, a reaction mixture was prepared with 50mM phosphate buffer pH 7.6, 3.2 mM $MgCl_2$, 1.5mM ATP, and 1.5 mM glycerol following the methods reported by Paasteris and de Saad (1998) and Nishihara et al., (1999). The system was incubated at 37 ºC for 5 minutes. Glycerol phosphate was converted to the trimethylsilyl ether using a large excess of HMDS/TCMS in pyridine for 60 minutes at 100 ºC. Results are reported as µmole glycerol phosphate produced per minute.

There was a significant effect of enzyme source on activity ($p<.05$). *B. stearothermophilus* had the highest activity ($p<.05$), as determined using mean separations with Least Significant Difference, and the other enzymes were not different one from another.

Glycerol kinase activity of enzymes from different sources

| | mean µmole GP/minute/mg protein |
|---|---|
| *Cellulomonas* | 24.2a |
| Amano 2 | 21.6a |
| *B. stearothermophilus* | 60.9b |
| *E. coli* | 29.2a |

The results were significantly lower than the rates expected at the optimal pH around 9. Still, there was quite strong measured activity and clearly the *Bacillus* enzyme had the greatest activity under the conditions assayed.

Glycerol phosphate oxidase (GPO)

This enzyme was studied in combination with the peroxidase and 4-chlorophenol indicator to simplify determination of enzyme activity. The oxidation products of 4-chlorphenol were followed by using a spectrophotometer at 540nm (Shimadzu 2101PC) using a 1cm quartz cell.

GPO enzymes (below) were obtained from Fluka (*Aerococcus*) and Sigma (*Streptococcus* and *Pediococcus*). Horseradish peroxidase (Type I) was obtained from Sigma. DL-α-glycerophosphate disodium was obtained from Sigma.

Streptococcus thermophilus
Pediococcus
Aerococcus viridans

Reactions were set up (2.0 ml) in 10mM Tris buffer (pH 7.6) containing 5mM α-glycerolphosphate, GPO (1mg), horseradish peroxidase (1 mg), and 10mM 4-chlorophenol. Tubes were incubated at 37 ºC for 10 minutes and then the absorbance was read at 510nm in a quartz cuvette (Shimadzu 2101PC). The results for duplicate determinations are shown below. A blank was used to zero the spectrophotometer which contained all of the reagents except GPO.

GPO activity of enzymes from different sources

| | absorbance at 15 minutes |
|---|---|
| *Streptococcus* | 0.21 ± .07 |
| *Pediococcus* | 0.36 ± .10 |
| *Aerococcus* | 0.09 ± .03 |

Despite the differences in the absorbances observed, there was no significant difference between the enzyme sources ($p=.07$) due to the relatively high variability observed. Because *Pediococcus* had a numerically higher value than the other enzymes, we decided to use this for the reagent strip.

Reagent strip evaluation

The enzymes selected based on prior experiments were *Mucor meihei* lipase, *Bacillus stearothermophilus* glycerol kinase, *Pediococcus* glycerolphosphateoxidase. Reagent films were obtained by dissolving 1g gelatin in 40ml hot distilled water. Upon cooling to ~45 ºC, the solution was mixed well, and enzymes and other reagents were added with vigorous mixing. The solutions were plated onto 20cmx20cm plastic sheets when the viscosity became high enough to produce about a 2mm thick, 5mm layer on the bottom of the sheet. The gelatin was allowed to dry at room temperature overnight. No reflective backing layer was present on the plastic sheets. Individual strips were hand cut from the master sheet.

The test strips were prepared and tested on artificially contaminated egg white samples in the range of 0.01% to 0.2% yolk contamination range in small samples that were incubated at 37 ºC in small tubes. Attempts to determine color changes with the test strips were unsuccessful. Parameters that were changed included the temperature of the gelatin where enzymes were added, the time of incubation (up to 30 minutes), levels of individual components were increased by factors of 5 individually and collectively. None of the alterations resulted in color change that was perceptible even at the highest level of yolk contamination examined. We are continuing with the work on this to try and find the conditions that will result in positive test strip performance. This will include changing the redox indicator, trying films with higher porosity, using multilayers with pH adjustment in each layer, altering the phosphorylation source and experiments on model emulsions to determine if specific inhibitors are present in contaminated egg whites that may prevent the enzymatic reactions from occurring.

References

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[2] Lay Summary

An enzymatic test strip procedure was evaluated for the rapid measurement of triglycerides in yolk-contaminated egg white to quantify the level of yolk contamination. Experiments were conducted to select the enzymes with the highest activity. Test strips were prepared combining 4 enzymes, other reactants and the indicator using gelatin as the solid support. Although the enzymes when tested separately were shown to have excellent activity, the tests with the reagent were not successful. Further work is required to develop a working prototype.