

**CONFORMATIONAL STABILITY, STABILIZATION AND APPLICATION  
OF PAPAINE IN ORGANIC MEDIA**

Summary of Ph.D. Thesis

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2009

**INTRODUCTION**

Enzymatic reactions in non-conventional media have recently received a great deal of attention. The use of enzymes in non-aqueous media has extended the scale of their practical applications and allowed the syntheses of drugs, flavours and polymeric, biologically active enantiomers that are difficult to obtain with conventional chemical catalysts. The advantages of using enzymes in industry include their high specificity, the mild reaction conditions, the non-toxicity and the biodegradation of the substrate and the product. The use of non-aqueous organic solvents as reaction milieu is associated with advantages such as the improved solubility of substrates and products, enhanced thermostability of the enzyme, a shift in the thermodynamic equilibrium to favour synthesis over hydrolysis, the suppression of many water-dependent side-reactions, and decreased microbial contamination, in addition to high levels of selectivity, specificity and catalytic rates under mild conditions. However, the exploitation of such advantages is often limited by the low stability and activity of biocatalysts in these systems.

Organic solvents can inactivate enzymes in several ways: Organic solvent molecules can interact with the biocatalyst, disrupting the secondary bonds in the native structure; they can strip the essential water molecules from the hydration shell and the structure of the enzyme; or they can interact with the active site of the biocatalyst, causing inactivation. The stabilization of enzymes is one of the most complex challenges in protein chemistry. A number of techniques have therefore been developed in order to reduce the inactivation, involving immobilization (Akrilex and Sorsilen), the use of

additives (ions or polyhydroxy compounds), chemical modification (aldehydes and anhydrides) and genetic engineering.

In organic media, it is possible to carry out many chemical reactions (syntheses of peptides and esters, esterification, transesterification, aminolysis, isomeration, etc.) through the use of hydrolytic enzymes.

Papain is a stable enzyme, one of the sulfhydryl proteases of *Carica papaya* latex. In view of its wide substrate specificity, papain is frequently utilized in the chemical synthesis of peptides and amino acid esters, though its catalytic activity, stability and structure in organic media were not reported in the earlier literature.

## OBJECTIVES

The goals of our work were to study the effects of water-miscible [ethanol, methanol, acetone, acetonitrile (ACN) and tetrahydrofuran (THF)] and water-immiscible (toluene, ethyl acetate and *n*-hexane) organic solvents with different chemical characters on the catalytic activity and stability of papain.

It was planned to follow the alterations in the secondary and tertiary structures of the enzyme by means of fluorescence spectroscopic and far- and near-UV circular dichroism (CD) measurements.

Organic solvents may cause reductions in enzyme activity. Accordingly, another goal was to enhance the stability of papain in aqueous organic solvents through the application of carbohydrates.

We decided to determine whether the presence of carbohydrates causes structural alterations in papain, and whether covalent interactions can occur between carbohydrates and enzyme molecules.

A further aim was to enhance the activity of papain via chemical modifications, using organic acid anhydrides (citraconic, maleic, succinic, acetic and propionic anhydrides).

The goal was to establish the heterogeneity of the chemical modifications. Thus comparisons were made of the native polyacrylamide gel electrophoretic mobilities of modified papain forms.

We set out to detect the number of modified amino groups after chemical modification and the changes in catalytic activity, efficiency, kinetic parameters ( $K_M$  and  $V_{max}$ ) and structure of papain.

The synthetic activities of the modified enzyme forms were compared in the production of *N*-acetyl-L-tyrosine ethyl ester.

## METHODS

### Assay of enzyme activity

The activity of papain was determined with casein and Z-Arg-7-amido-4-methylcoumarin. The measurements were performed at 25 °C.

### Stability tests

The stability of papain was studied at organic solvent contents in the interval 0–90 v/v%. Enzyme solution was added to the incubation mixture, the samples were incubated for 0–120 min and the activities were then determined.

### Chemical modification

Papain was modified with acetic, propionic, citraconic, maleic or succinic anhydride in 0.1 M phosphate buffer at pH 8.0 and 25 °C. The enzyme samples were then gel-filtered to remove the unreacted modifier molecules.

### Determination of free amino groups

The number of free amino groups was determined spectrophotometrically, using 2,4,6-trinitrobenzenesulfonic acid. The number of modified amino groups was calculated from the difference in the numbers of free amino groups in the unmodified and modified enzyme forms.

#### **Native polyacrylamide gel electrophoresis**

The degrees of homogeneity of unmodified and modified papain were investigated by means of native polyacrylamide gel electrophoresis. The assay was performed in 12% resolving gel associated with 4% stacking gel.

#### ***N*-acetyl-L-tyrosine ethyl ester synthesis**

The efficiencies of the modified papain forms were compared in the synthesis of *N*-acetyl-L-tyrosine ethyl ester in 96 v/v% ethanol. The amount of the product was determined from the amount of *N*-acetyl-L-tyrosine remaining after a 24-h incubation at 25 °C.

#### **Fluorescence spectroscopy**

The samples were treated according to the stability tests. For fluorescence of the Trp content, excitation was performed at 295 nm and the emission spectra were recorded in the interval 300–400 nm after 20–120 min at 25 °C.

#### **Circular dichroism spectroscopy**

CD spectra were recorded in the far-UV range, from 190 to 250 nm, and in the near-UV range, from 250 to 300 nm, at 25 °C. The samples were treated according to the stability tests. The measurements were carried out in the concentration range 0–90 v/v% after a 30- or 120-min incubation.

### **SUMMARY OF RESULTS**

1. The effects of the organic solvents on the activity of papain in aqueous solutions were different, but the data correlated well with the hydrophobicity

(*log P*) of the solvents. We clarified that the catalytic activity of the enzyme was preserved with decreasing hydrophobicity of the solvent. While papain in aqueous ethanol, methanol, acetone or ACN retained much of its catalytic activity, *n*-hexane, toluene, ethyl acetate and THF caused dramatic reductions in activity.

2. The fluorescence properties of the Trp residues were studied in aqueous ethanol, methanol, ACN and THF. Our results showed that aqueous ethanol and methanol did not alter the structure of papain. However, in aqueous ACN and THF, more significant changes were observed in the structure of the enzyme. The far-UV CD spectra in aqueous ethanol and ACN indicated increases in the helical structural elements. Near-UV CD measurements revealed that only aqueous THF caused dramatic alterations in the tertiary structure of papain.
3. In THF, the residual activity of papain was decreased significantly after 30 min. The THF-induced inactivation of papain could be compensated by the addition of carbohydrates under basic conditions. The stabilizing effects of polyols on the activity of papain decreased in the sequence D-ribose > D-fructose > D-glucose > D-saccharose > D-raffinose. Our results also confirmed that the stabilizing effects of carbohydrates correlated well with the number of their hydroxy groups.
4. The near-UV CD spectra and Trp fluorescence parameters of papain were investigated in the presence of carbohydrates. The changes in the fluorescence parameters suggested a THF-induced denaturation. Ribose and fructose in aqueous THF resulted in significant changes in the fluorescence

parameters as compared with those observed in THF. In the near-UV CD spectrum of papain, a considerable decrease was observed in THF as compared with that in water. The increased spectral intensities in the presence of fructose and ribose in aqueous THF indicate that these sugars exert protective effects on the tertiary structure of papain. The carbohydrates may be incorporated into the hydration shell of the enzyme. Moreover, reducing sugars such as ribose and fructose are known to have the ability to react non-enzymatically in their open chain form with free amino groups of proteins, producing Schiff bases.

5. Chemical modification of the primary amino groups of enzymes (amino terminal and lysine residues) with organic acid anhydrides is another useful strategy for enhancing the stability of biocatalysts. Modifications were carried out with acetic, propionic, citraconic, succinic and maleic anhydrides. Monocarboxylic acid anhydrides (acetic and propionic anhydrides) neutralized the positive charge on the lysine residues, whereas dicarboxylic acid anhydrides (citraconic, succinic and maleic anhydrides) replaced the positive charges on the lysines with negatively charged carboxyl groups in the enzyme structure. On the use of acid anhydrides, the majority of the 11 primary amino groups of papain were modified. The modified enzyme forms exhibited heterogeneous patterns, containing 2 or 3 bands, because the primary amino groups of the papain molecules were not acylated equally, and consequently there may be some variances in modification between the enzyme molecules.

6. In comparison with unmodified papain, the modified enzyme exhibited significant increases in catalytic activity in phosphate buffer (pH 8.0).

Increases in the catalytic activity of modified papain were also measured in aqueous ethanol. The catalytic activities of the forms of papain modified by treatment with monocarboxylic acid anhydrides were higher than those of the enzyme forms modified with dicarboxylic acid anhydrides. The stabilizing effects in aqueous ACN and THF were more moderate.

7. The Trp fluorescence parameters of papain suggested that only light structural changes were observed in citraconyl and propionyl papain in aqueous ethanol. The near-UV CD spectra showed that in aqueous ethanol the modified enzyme preserves a native structure similar to that detected for the unmodified enzyme in buffer.

8. A model reaction for the synthesis of N-acetyl-L-Tyr ethyl ester was carried out in aqueous ethanol in order to investigate the efficiencies of the modified papain forms. The modifications increased the conversion of the ethyl ester synthesis. Papain modified with a monocarboxylic acid anhydride proved more effective than the enzyme modified with a dicarboxylic acid anhydride. The results indicate that neutralization of the lysine residues with a monocarboxylic acid anhydride yields a more effective enzyme than does modification with a dicarboxylic acid anhydride.

In the course of our experiments, the unfavourable effects of organic solvents on the stability of papain were diminished by means of the addition of carbohydrates and chemical modifications with organic acid anhydrides. The results may help shed light on the effects of organic solvents on the activities and stabilities of enzymes and on the activity–structure relationship with a view to improving their industrial applications.

## PUBLICATIONS

### a) Publications directly related to the subject of the theses:

1. **A. Szabó**, M. Kotormán, I. Laczkó, L.M. Simon (2006) Spectroscopic studies of stability of papain in aqueous organic solvents. *J. Mol. Catal. B: Enzym.* **41**. 43–48 (IF: 2.149)
2. **A. Szabó**, M. Kotormán, I. Laczkó, L.M. Simon (2009) Influence of carbohydrates on the stability of papain in aqueous tetrahydrofuran mixture. *J. Chem. Tech. Biotechnol.* **84**. 133–138 (IF: 1.426)
3. **A. Szabó**, M. Kotormán, I. Laczkó, L.M. Simon (2009) Improved stability and catalytic activity of chemically modified papain in aqueous organic solvents. *Process Biochem.* **44**. 199–204 (IF: 2.336)

### b) Posters and papers in connection with the theses:

1. **A. Szabó**, M. Kotormán, I. Laczkó, J. Nemcsók, L.M. Simon (2005) 30<sup>th</sup> FEBS Congress and 9<sup>th</sup> IUBMB Conference. The FEBS Journal. **272**. Supplement 1. Abstr. G2-117P. Budapest. Hungary
2. **A. Szabó**, M. Kotormán, L.M. Simon (2007) 7<sup>th</sup> International Conference on Protein Stabilization. Abstr. 41. Exeter. UK
3. **A. Szabó**, M. Kotormán, I. Laczkó, L.M. Simon (2008) MBKE 2008. évi vándorgyűlése. Abstr. P-62. Szeged. Hungary

### c) Other publications:

1. M. Kotormán, I. Laczkó, **A. Szabó**, L.M. Simon (2003) Effects of Ca<sup>2+</sup> on catalytic activity and conformation of trypsin and  $\alpha$ -chymotrypsin in aqueous ethanol. *Biochem. Biophys. Res. Commun.* **304**. 18–21 (IF: 2.836)
2. L.M. Simon, M. Kotormán, **A. Szabó**, G. Garab, I. Laczkó (2004) Effects of polyethylene glycol on stability of  $\alpha$ -chymotrypsin in aqueous ethanol solvent. *Biochem. Biophys. Res. Commun.* **317**. 610–613 (IF: 2.904)
3. L.M. Simon, M. Kotormán, **A. Szabó**, J. Nemcsók, I. Laczkó (2007) The effects of organic solvent/water mixtures on the structure and catalytic activity of porcine pepsin. *Process Biochem.* **42**. 909–912 (IF: 2.336)

### d) Other posters and papers

1. L.M. Simon, M. Kotormán, **A. Szabó**, I. Laczkó (2004) 6<sup>th</sup> International Conference on Protein Stabilization. Abstr. 54. Bratislava. Slovakia
2. M. Kotormán, Z. Terbe, **A. Szabó**, G. Garab, M.L. Simon (2005) 30<sup>th</sup> FEBS Congress and 9<sup>th</sup> IUBMB Conference. The FEBS Journal. **272**. Supplement 1. Abstr. G2-111P. Budapest. Hungary