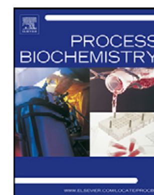
Process Biochemistry 47 (2012) 47–56

Contents lists available at SciVerse ScienceDirect

Process Biochemistry

journal homepage: www.elsevier.com/locate/procbio



Investigation of the structure and proteolytic activity of papain in aqueous miscible organic media

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ARTICLE INFO

Article history:
Received 6 September 2011
Received in revised form
30 September 2011
Accepted 7 October 2011
Available online 19 October 2011

Keywords:
Papain
Aqueous–organic media
Catalytic stability
Structural stability
Thermal stability
Fluorescence spectroscopy
ATR-FTIR spectroscopy

ABSTRACT

The stability of papain was studied in aqueous–organic mixtures by means of residual proteolytic activity along with various spectroscopic analyses (fluorescence and ATR-FTIR combined with isotopic exchange with D_2O). The investigated systems contained 1 or 10% (v/v) of an aqueous buffered solution (pH 8.0) in acetonitrile (ACN), methanol (MeOH) or dimethyl formamide (DMF). The results evidenced that papain retained almost all its catalytic activity after 24 h of incubation in the presence of ACN, and a more compact conformation of the enzyme was detected. Papain suffered an important loss of enzymatic activity (ca. 80%) after 24 h incubation in MeOH although, no global conformational change and minor secondary structure rearrangements were detected. This observation suggests that somehow the active site region was altered. On the other hand, papain suffered a complete inactivation when exposed to those media containing DMF. Fluorescence analyses revealed that an irreversible conformational change took place after 24 h incubation, and a moderate increase in β-sheet and β-turn structures was the most relevant finding when secondary structure was analyzed. The evidences demonstrated that the organic solvents induce a more rigid and compact structure of papain regardless of the organic:buffer ratio investigated. In turn, these modifications affect the active catalytic site in the particular case of MeOH and DMF. These findings were in agreement with the thermo-stability of the enzyme performed after heating at 353 K in all the studied media, that is the presence of ACN did not substantially affect the secondary structure of papain. Nevertheless, the α-helix domain demonstrated to be less thermally stable than the β-sheet domain, turning into aggregated structures after heating, especially in the presence of MeOH and DMF.
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1. Introduction

Papain (EC 3.4.22.2) is a cysteine endopeptidase that is found in latex of the papaya plant (*Carica papaya*). It is widely used in food, detergent, textile and pharmaceutical industries due to its versatility and easy production. However, when a substantial quantity of papain with high purification degree is needed, a wild-type recombinant form of the enzyme would be recommended in order to avoid shortcomings such as the use of a lot of raw material, dependence on the climatic conditions for growth, and methods used for its extraction and purification among others. Cloning and expression of papain has been made in many different systems; however, the production of recombinant papain in a sustainable fashion is still under study [1,2].

In this context, the influence of the operational conditions of industrial processes on the enzymatic activity, the effect of different factors (such as temperature, and exposure to stabilizing and denaturing agents, among others) on its conformation and function are currently a topic of intensive studies. These investigations are mainly based on the fact that papain is a model cysteine peptidase, since its structure is well established and therefore, a considerably amount of data are available [3–6].

This enzyme catalyzes the synthesis of amides and esters, hydrolysis of macromolecules, and asymmetric hydrolysis in a variety of reaction media such as aqueous, organic, biphasic organic–aqueous media and ionic liquids. In this context, papain is suitable as a biocatalyst in the synthesis of fine chemicals and pharmaceuticals and an interesting alternative to make greener processes. The conditions in which these reactions take place might affect, to a more or less extent, papain structure and thus, its catalytic activity. Several studies revealed that the exposure to different environments, denaturing and stabilizing agents causes many folding/unfolding transition states that affect its secondary

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doi:10.1016/j.procbio.2011.10.003

Table 1
Summary of most relevant investigations of the bio-catalytic uses of papain with special focus on: type of reaction (only homogeneous were considered); substrates involved in the process; organic, aqueous or mixed reaction media; investigation of the secondary and/or tertiary structure of the enzyme and the application of isotopic exchange for Amide I investigation.

Type of reaction	Substrates	Medium	Structure investigation	Isotopic exchange	Ref
Protected dipeptide Boc-Gly-Phe-OMe synthesis	Boc-Gly-OH Phe-OMe Phe-OBz	Trichloroethylene, CCl_4, ciclopentanone, benzene, toluene, *n*-heptane, Tween 80	No	No	[7]
Z-Gly-Phe-NH_2 synthesis	Z-Gly-OH Phe-NH_2	Tert-butanol with solid acid-base buffers	No	No	[8]
None	None	Aqueous sodium dodecyl sulfate	Circular dichroism, fluorimetry, FTIR, UV–vis, NMR, dynamic light scattering	Yes	[9]
Hydrogenation	Methyl 2-acetamidoacrylate	Aqueous buffer (pH 7)	No	No	[10]
Hydrolysis	Casein	Aqueous, ethanol; 1,4 dioxane; acetonitrile, tetrahydrofuran	Fluorescence, circular dichroism	No	[11]
Asymmetric hydrolysis	D,L-*p*-Hydroxyphenylglycine methyl ester	1-Alkyl-3-methyl-imidazolium cations ionic liquids	ATR-FTIR, fluorescence	No	[12]
Synthesis of Z-L-aminoacyl-antipyrine amides	Z-protected amino acid esters 4-Aminoantipyrine	Aqueous–organic, biphasic media	No	No	[13]
Hydrolysis	Z-Arg-AMC	0–90% (v/v) aqueous ethanol, acetonitrile, tetrahydrofuran	Fluorescence, circular dichroism	No	[14]
Hydrolysis	Casein	*n*-Propanol–NaClaq, hexane/water, PEG/phosphate biphasic	No	No	[15]
Hydrolysis	Bovine serum albumin (BSA), ovalbumin, casein, *N*-benzoyl-L-arginine ethyl ester	Aqueous buffered	No	No	[16]

and tertiary structures [5–8]. In this context, Table 1 summarizes the most relevant investigations concerning the uses of papain as a biocatalyst in the homogeneous fashion along with the substrates involved in the process, nature of the reaction media, and investigation of the secondary and/or tertiary structure of the enzyme [9–18]. Focusing on infrared studies, the application of isotopic exchange for Amide I investigation is highlighted.

A detailed literature search also demonstrates that there are few investigations presenting structure-stability-activity correlations for the application of papain as biocatalyst in reactions occurring in mixtures of aqueous–organic media. Simon and coworkers demonstrated that the tertiary structure of papain is influence by water-miscible solvents and hence its activity [13]. Further investigations of the authors proved that the tolerance towards organic solvents is increased when the enzyme is chemically modified with monocarboxylic and dicarboxylic acids [16]. More recently, Wang and coworkers investigated the modifications of the protein conformation of papain due to the impact of ionic liquids [14]. The authors reported the second derivative of the ATR-FTIR spectra of the Amide I region of papain as a sensitive probe of protein conformation. Ghosh investigated conformational modifications due to the interactions of papain with an anionic surfactant in aqueous medium by analyzing the secondary structure through FTIR after the exchange of the molecules of H_2O by D_2O [11]. In this sense, the isotopic exchange avoids the strong O–H bending absorption at 1644 cm^{-1} of H_2O which overlaps with the infrared signal of the Amide I. The O–D bend is shifted about 400 cm^{-1} to lower frequency as compared to the O–H bend, thus leaving an useful window free of absorptions in the Amide I region [19]. The usefulness of ATR-FTIR in combination with H_2O/D_2O exchange for secondary structure determination of papain was firstly described by Goormaghtigh et al. [20].

The present investigation focuses on the determination of the structure and stability of papain extracted from papaya latex using methanol (MeOH), acetonitrile (ACN) and dimethylformamide (DMF) containing 1% and 10% of added water. The behavior of the protease in aqueous buffer of pH = 8.0 was also examined and established as the reference condition, since the conformation that the enzyme exhibits at this pH is considered as the most favorable for peptide synthesis in the presence of organic solvents [21]. In this context, biological stability was evaluated through the determination of its proteolytic activity. Additionally, intrinsic and extrinsic fluorescence spectroscopy and attenuated total reflexion infrared spectroscopy (ATR-FTIR) were used in order to obtain evidences of effect of the solvents both at the surface and at the bulk of the enzyme. Finally, the effect of the solvents on the thermal stability of papain was also addressed through *in situ* ATR-FTIR analysis.

2. Materials and methods

2.1. Chemicals

Papain was purified to mass spectrometry degree from dry latex of *Carica papaya* fruits obtained in Jujuy, Argentina, as described previously [22,23]. Casein (Hammarsten type) from bovine milk, tris-(hydroximethyl)-aminomethane (Tris), cysteine, 8-aniline-1-naphthalene-sulfonic acid (ANS) and *N*-acetyl-L-Trp ethyl ester (ATrEE) were Sigma Aldrich products. Coomassie Brilliant Blue G-250 was obtained from Bio-Rad. Acetonitrile (ACN) and methanol (MeOH) were obtained from Baker. *N*,*N*-dimethylformamide (DMF) was from Merck. Additional chemicals were obtained from commercial sources and were of the highest purity available.

2.2. Stability assays

The mixtures of organic solvent and buffered aqueous solution were composed by 1 and 10% of aqueous boric acid-sodium borate buffer 0.1 M pH 8.0, and 99 and 90% of a miscible organic solvent such as, ACN, MeOH and DMF. Papain (0.4 mg) was contacted with 1.00 ml of each of those organic solvent-buffer mixtures and incubated at 37 °C with orbital shaking (180 rpm) during 1 and 24 h. Then, the samples were centrifuged (10 min, 4 °C, 3600 × g) and the supernatant containing the organic–aqueous mixture was discarded. Each pellet was dried under vacuum and redissolved in 0.5 ml of Tris–HCl buffer 0.1 M pH 8.0, and the residual activity was tested using casein as substrate according to the method reported by Priolo et al. [24]. Protein concentration of each sample was determined according to Bradford's method [25]. Specific enzymatic activity was expressed in an arbitrary unit (Ucas/mg

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