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Short communication

The effects of organic solvent/water mixtures on the structure and catalytic activity of porcine pepsin

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Abstract

The effects of aqueous solutions of ethanol, acetonitrile and 1,4-dioxane in the concentration range 10–90% (v/v) on the activity of porcine pepsin were studied. The enzyme retained its activity in aqueous ethanol and aqueous acetonitrile with increasing organic solvent concentration up to 60%, and in aqueous 1,4-dioxane up to 30%, but thereafter a considerable decrease in activity was observed. The changes caused in the catalytic activity by the water-miscible organic solvents may be related to structural changes, which were followed by means of intrinsic fluorescence and circular dichroism spectroscopy measurements.

Keywords: Porcine pepsin; Enzyme activity; Aqueous organic solvents; Intrinsic fluorescence; CD spectroscopy

1. Introduction

The use of enzymes in organic solvents has extended the scale of their practical applications and allowed the syntheses of polymeric, biologically active enantiomers that are difficult to obtain with conventional chemical catalysts. Biocatalysis in organic media has been a subject of intensive basic and application-oriented research [1–3]. Although the ability of enzymes to act as selective catalysts for a broad spectrum of organic reactions has been known for many years, their application is rare because of the inappropriate stabilities of the biocatalysts. The solvents influence the catalytic properties and stabilities of enzymes to a considerable extent [4,5]. The stability of enzymes is one of the most difficult problems in protein chemistry, in consequence of the great number of factors involved and the lack of methods permitting an evaluation of their individual contributions. Protein molecules in aqueous solutions are surrounded by a hydration shell, composed of water molecules attached to the protein surface. If an organic solvent is also present, the solvent molecules tend to displace the water molecules both from the hydration shell and from the interior of the protein, thereby distorting the interactions responsible for maintaining the native conformation of the enzymes. Inactivation of enzymes in different organic solvents has been reported, but the mechanism of inactivation at a molecular level remains unclear. Some enzymes do not display any significant structural changes during inactivation, and local active site effects might be responsible for the loss in activity [6,7].

Pepsin (EC 3.4.23.1) is a well-known, monomeric aspartic protease possessing two, mainly β-protein domains, with a high percentage of acidic residues [8]. Proteases (trypsin and α-chymotrypsin) are widely used for peptide and amino acid ester syntheses in different organic solvents [9,10]. It is somewhat surprising, therefore, that the literature furnishes comparatively few data concerning the stability and applicability of pepsin in organic solvents. We earlier studied the effects of water-miscible organic solvents [ethanol, 1,4-dioxane and acetonitrile (ACN)] at different concentrations on the kinetic parameters and conformational stability of hydrolytic enzymes, and found significant changes in the secondary structures of these enzymes [11,12].

The aim of the present work was to study the effects of these three organic solvents on the catalytic activity of porcine pepsin. The alterations in the structure of the enzyme were followed by

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means of circular dichroism (CD) and intrinsic fluorescence spectroscopy measurements.

2. Materials and methods

2.1. Materials

Pepsin (from the porcine pancreas) was from Sigma–Aldrich. Its specific activity was 2950 U per milligram of protein. All other chemicals were reagent grade products from Spektrum.

2.2. Assay of enzyme activity

For the measurement of pepsin activity, haemoglobin was used as substrate [13]. The reaction mixture (1.2 ml) contained 3.3 mg haemoglobin and 0.1 mg pepsin dissolved in 50 mM HCl. After incubation for 5 min at pH 2 and 25 °C, the reaction was terminated by the addition of 2 ml 5% (w/v) trichloroacetic acid, and the undigested protein was precipitated. After centrifugation for 10 min at 14,000 rpm, the supernatants containing the acid-soluble fragments of haemoglobin were estimated spectrophotometrically at 280 nm or with the Folin Ciocalteu reagent [14].

2.3. Stability tests

The stability tests in aqueous organic solvents were performed at 10–90% (v/v) concentrations of the organic solvents with an enzyme concentration of 0.1 mg ml^{-1} at pH 2.0 and 25 °C. The samples were incubated for 20 min, 200 μl aliquots were then withdrawn and the residual activities of the enzyme were determined by using the standard method described above. Experiments were performed in triplicate and the average values are presented.

2.4. Fluorescence spectroscopy

The fluorescence spectrum of pepsin was monitored on a Hitachi F-2500 FL spectrofluorimeter. Enzyme samples were previously incubated for 20 min and the final protein concentration was 0.1 mg ml^{-1}. Three spectra were accumulated and averaged for each sample. For the fluorescence of the tryptophan (Trp) residues in pepsin, excitation was performed at 292 nm and emission spectra were recorded in the interval 300–450 nm, with slit widths of 2.5 and 5 nm for excitation and emission, respectively [15].

2.5. CD measurements

CD spectra were recorded in the near-UV range (from 250 to 300 nm) in a 1 cm cell on a Jobin–Yvon Mark VI dichrograph at 25 °C. Four spectra were accumulated and averaged for each sample. The concentrations of the protein solutions were adjusted to 0.55 mg ml^{-1}. Mean residue ellipticity, $[\Theta]_{MR}$, was expressed in cm^2 $dmol^{-1}$, using a mean residue weight of 110.

3. Results

3.1. Enzyme activity

Fig. 1 depicts the effects of different concentrations of aqueous ethanol, 1,4-dioxane and ACN solutions on the activity of pepsin after incubation for 20 min at pH 2 and 25 °C. Some loss in catalytic activity was observed as the concentration of ethanol and ACN was increased up to 60% (v/v), but thereafter the enzyme activity decreased considerably. In 1,4-dioxane, the enzyme activity was relatively well preserved only up to an organic solvent concentration of 30% (v/v), above which it decreased dramatically, and it was fully lost at an organic solvent content of 60%.

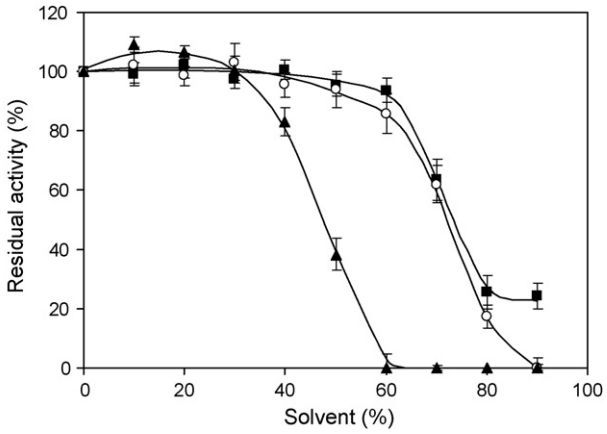

Fig. 1. Effects of different concentrations of aqueous acetonitrile (○), 1,4-dioxane (▲) and ethanol (■) solutions on the activity of pepsin. The enzyme activity measured in water as solvent was taken as 100%. Experiments were carried out at 25 °C, with incubation for 20 min. Enzyme concentration: 0.1 mg ml^{-1}.

3.2. Fluorescence measurements

The fluorescence of proteins is a very sensitive indicator of the microenvironment of the Trp residues. The fluorescence properties of the Trp in pepsin were studied in aqueous ACN, ethanol and 1,4-dioxane. Both the protein conformation in the

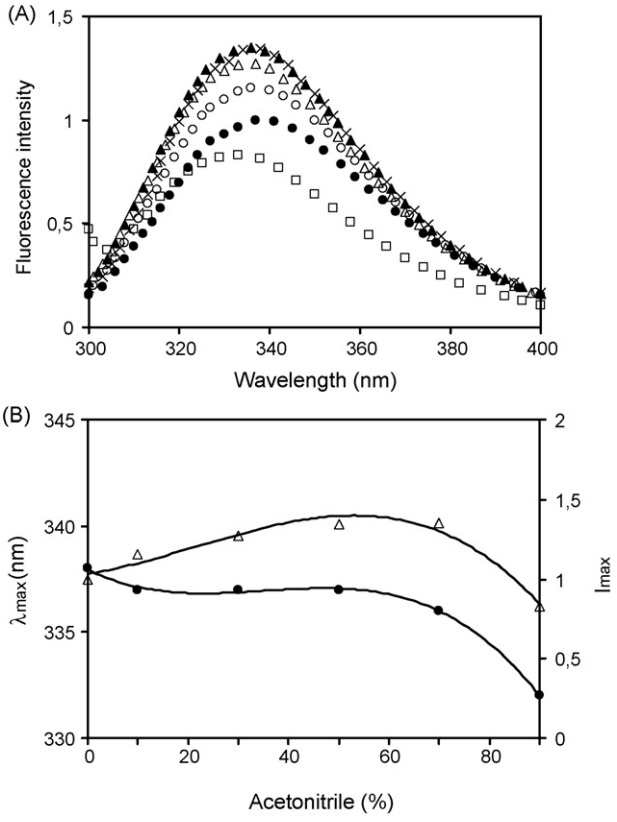

Fig. 2. (A) Fluorescence spectra of pepsin at different aqueous acetonitrile concentrations: 0 (●), 10% (○), 30% (△), 50% (▲), 70% (×), 90% (□). (B) Dependence of λ_{max} (●) and I_{max} (△) of pepsin fluorescence on acetonitrile concentration. The measurements were performed after incubation for 20 min at 25 °C with 0.1 mg ml^{-1} pepsin. Excitation wavelength: 292 nm; slit: 2.5 nm. Emission wavelength: 300–450 nm; slit: 5 nm.