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Stabilization of α-chymotrypsin in aqueous organic solvents by chemical modification with organic acid anhydrides

Márta Kotormán[a], András Cseri[a], Ilona Laczkó[b], L. Mária Simon[a,*]

[a] Department of Biochemistry and Molecular Biology, Faculty of Science and Informatics, University of Szeged, Középfasor 52, H-6726 Szeged, Hungary
[b] Institute of Biophysics, Biological Research Center of Hungarian Academy of Sciences, Temesvári krt. 62, H-6726 Szeged, Hungary

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ABSTRACT

When the primary amino groups of α-chymotrypsin were modified with acetic, propionic, succinic, citraconic and phthalic acid anhydrides, the modifications enhanced the stability of the enzyme in 60% aqueous solutions of 1,4-dioxane, ethanol and acetonitrile. The acetylation of the amino groups resulted in the lowest stabilization, but the citraconylated, propionylated and succinylated forms of α-chymotrypsin exhibited increased stabilities in all the aqueous organic solvents studied. The modification of α-chymotrypsin with phthalic anhydride was accompanied by very extensive activation. The near-UV circular dichroism spectroscopic measurements did not indicate significant structural changes in the acylated forms of α-chymotrypsin, with the exception of the phthalic anhydride-modified enzyme. The spectral changes in the phthalic anhydride-modified enzyme suggest enhanced interactions between the aromatic chromophores and alterations in the disulfide contribution. The improvement in stability may be related to the modifications caused by the reorientation and the increased interactions of the aromatic side-chains, particularly in the case of the phthalic anhydride derivative.

1. Introduction

The application of biocatalysts in non-conventional media has undergone significant development in the past two decades, and numerous reactions have been introduced and optimized for synthetic applications. In contrast with aqueous enzymology, biotransformations in water–organic solvent mixtures offer unique, industrially attractive advantages, such as changes in the enantioselectivity of reactions, reversal of the thermodynamic equilibrium of hydrolyses, suppression of side-reactions, increased thermostability and resistance to microbial contamination [1]. The removal of enzymes from their natural environs results in reduced reaction rates and low stabilities. The methods used to increase enzyme activity and stability in organic solvents range from simple to complex: protein engineering, such as site-directed mutagenesis and directed evolution, which alter the enzyme specificity and enhance the activity in organic solvents [2–4]. Immobilization, solvent engineering, the use of additives and chemical modifications are commonly used techniques. The chemical modification of amino acid side-chains allows the introduction of an almost unlimited variety of groups. α-Chymotrypsin as a model enzyme has been modified with both hydrophobic and hydrophilic compounds in order to stabilize the enzyme against denaturation both in aqueous buffer and in aqueous organic media [5,6]. Chemical modification has now reemerged as a complementary approach to site-directed mutagenesis and directed evolution [7,8].

The aims of our present work were to modify the primary amino groups of α-chymotrypsin with acetic, propionic, succinic, citraconic and phthalic anhydrides so as to enhance the conformational stability of the enzyme, and to compare the stabilities of the acylated enzyme forms in aqueous organic solvents. The effects of decrease of the number of positive charges, the presence of negative charges instead of positive charges, and the introduction of an aromatic ring into the protein structure on the enzyme activity/stability were studied.

2. Materials and methods

2.1. Materials

The synthetic substrates N-acetyl-L-tyrosine ethyl ester (ATEE) and α-chymotrypsin (EC 3.4.21.1; type II from the bovine pancreas; specific activity 50 U/mg) were from Sigma–Aldrich. Phthalic anhydride was obtained from Fluka. Acetic, propionic, succinic and citraconic anhydrides and all other chemicals used were reagent grade products of Sigma.

* Corresponding author. Tel.: +36 62 544105; fax: +36 62 544887.
E-mail address: lmsimon@bio.u-szeged.hu (L.M. Simon).

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2.2. Assay of enzyme activity

For the measurement of α-chymotrypsin activity, ATEE was used: the decrease in absorbance at 237 nm was followed in a reaction mixture (3 ml) containing 45 mM Tris/HCl (pH 7.0) and 1 mM ATEE [9]. The reactions were initiated by the addition of 200 μl enzyme solution at a concentration of 0.04 mg/ml.

2.3. Stability tests

The stability tests in organic solvents were performed in 0.01 M KCl solutions with an enzyme concentration of 0.04 mg/ml at 25 °C. The samples were incubated for appropriate periods of time, aliquots were then withdrawn and the residual activities of the enzymes were determined by using the standard method described above. Results of three independent experiments are presented in the figures. The standard deviations of experimental values were within 5%.

2.4. Chemical modification

Chemical modification with the different anhydrides was carried out according to Mozhaev et al. [10] with some modifications. A solution (0.5 ml) of the anhydride (10–800 mM) in dimethyl sulfoxide was added dropwise at 0 °C to 4 ml of 40 μM α-chymotrypsin in 0.05 M phosphate buffer (pH 8.0) with stirring. The pH of the reaction mixture was kept constant by the addition of 1 M KOH solution. The reaction was allowed to proceed for 1 h. The solution was then fractionated by filtration on Sephadex G-25, with 0.1 M KCl as eluent, to remove the excess reagent. The degree of modification was calculated from the number of amino groups in the modified enzyme form which reacted with trinitrobenzenesulfonic acid as compared with the unmodified enzyme [11].

2.5. Circular dichroism (CD) measurements

Circular dichroism spectra were recorded in the near-UV range 250–300 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 concentration of the protein solutions in the measurements was adjusted to 0.3 mg/ml. Mean residue ellipticity $[\Theta]_{MR}$, was expressed in deg cm^2 dmol^{-1}, using a mean residue weight of 110.

3. Results and discussion

Five organic acid anhydrides (acetic, propionic, succinic, citraconic and phthalic anhydride) were used to study the effects of chemical modification of the primary amino groups of α-chymotrypsin on the stability and activity in aqueous organic solvents. α-Chymotrypsin contains 14 lysyl ε-amino and 3 N-terminal α-amino groups. From X-ray crystallographic studies, it is known that the amino groups are situated on the surface of the enzyme [12]. The modifications with acetic and propionic anhydrides resulted in the acylation of 9 or 12 of the amino groups, together with a decrease in the number of positive side-chains on the α-chymotrypsin, since these molecules contain only one carboxylic group. With citraconic anhydride 12, and with succinic anhydride 14, but with phthalic anhydride only 1 amino group was modified. These molecules contain 2 carboxylic groups; accordingly the positive charges of the amino groups on the enzyme were converted into negative ones, and with the phthalic anhydride modification an aromatic side-chain was introduced into the enzyme structure.

The effects of different concentrations of ethanol (10–90%) on the activities of the modified α-chymotrypsin forms were studied at

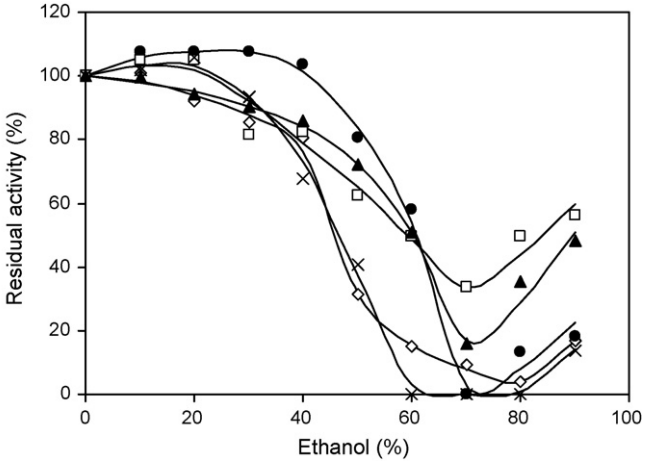

Fig. 1. Effects of different concentrations of aqueous ethanol on the activities of modified α-chymotrypsin forms after incubation for 20 min at 25 °C. Acetyl (▲), propionyl (●), citraconyl (◊), succinyl (□) and non-modified enzyme (×). Enzyme concentration: 0.04 mg/ml. The starting activities measured in 0.01 M KCl (acetyl 1.3 U/ml, propionyl 1.17 U/ml, citraconyl 1.05 U/ml, succinyl 0.85 U/ml and non-modified 1.23 U/ml) were taken as 100%.

25 °C after incubation for 20 min. The native enzyme lost its activity in 60% ethanol, whereas the modified enzymes preserved ~30–50% of their activities even in 90% ethanol (Fig. 1). The tolerance of the modified enzymes to higher concentrations (>50%) of ethanol was more pronounced, which might be important in the application of these enzyme forms in organic syntheses in solvents with low water contents. Similar results were found in acetonitrile and 1,4-dioxane.

The stabilities of the modified α-chymotrypsin forms in 60% ethanol, acetonitrile and 1,4-dioxane are depicted in Fig. 2A–C. Acetylation of the amino groups resulted in the lowest stabilization, but the citraconylated, propionylated and succinylated forms of α-chymotrypsin exhibited higher stabilities in all of the studied aqueous organic solvents.

The enzyme modified with phthalic anhydride displayed unexpected behaviour. In the presence of 60% ethanol, 1,4-dioxane or acetonitrile, extensive activation was observed (Fig. 3A). The activation in 60% acetonitrile, 60% 1,4-dioxane and 60% ethanol attained 300%, 236% and 220%, respectively. This activation developed within 30 min at 25 °C. [It was also observed at lower organic solvent concentrations, but it then took a longer time. For activation of the phthalic anhydride-modified enzyme, 1- or 2-propanol can also be used, but no activation was found in tetrahydrofuran or methanol (data not shown).] In 0.01 M KCl at 40 °C, activation of the enzyme modified with phthalic anhydride was likewise observed, but it took at least 60 min. However, in the presence of 3 M urea in 0.01 M KCl at 25 °C, the activation was higher and took 30 min (Fig. 3B). In aqueous buffer, the acylation of α-chymotrypsin with *o*-phthalic anhydride hardly increases the thermal stability of the enzyme [10]. Phthalic anhydride-modified horseradish peroxidase displayed increased stability in methanol, acetonitrile and dimethylformamide, with improved catalytic properties, related to the changes in the conformation of the enzyme [13].

Activation of the modified α-chymotrypsins with both hydrophilic and hydrophobic reagents was observed at 20–60% ethanol and dimethylformamide [5]. Phthalic acid-modified papain exhibited enhanced catalytic properties, and resistance to thermal and autolytic inactivation [14]. Papain modified with mono- (acetic, propionic) or dicarboxylic acid anhydrides (citraconic, maleic, or succinic) resulted in higher catalytic activity and stability both in water and in some aqueous organic solvents [15]. The introduction of a bulky benzene ring attached to the lysine side-chain provides a new opportunity for the occurrence of hydrogen bonding, thereby

preventing the unfolding and denaturation of the enzyme. The alteration in the charge conditions should introduce an increase in the extent of electrostatic repulsion within the polypeptide chain, contributing to the conformation and stability of α-chymotrypsin. The activation and higher stability of the phthalic acid-modified enzyme may in theory arise from the decrease in autolysis and the higher conformational stability. During the 2-h experiments, autolysis was not observed for either the native or the modified enzyme. The concerted effect of the above-mentioned small local changes in the enzyme structure is probably responsible for the enhancement of the catalytic activity and the stability of the modified enzyme form.

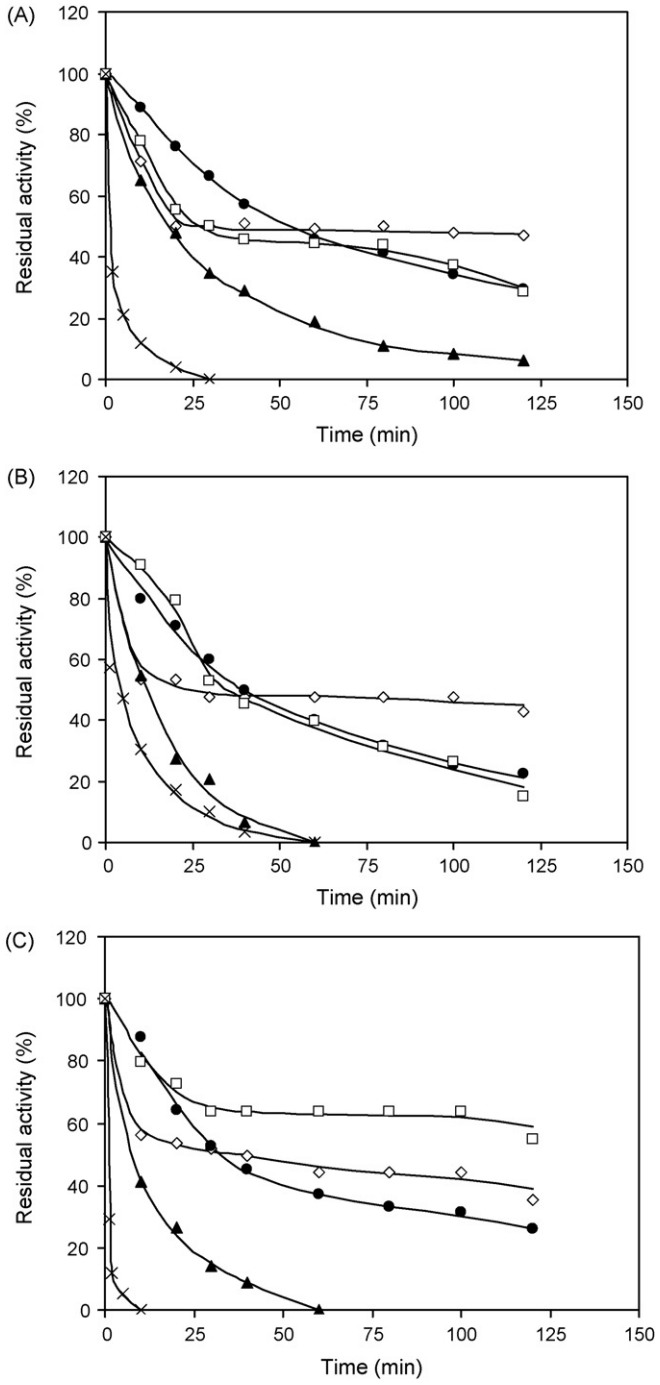

Fig. 2. Stabilities of α-chymotrypsin modified with acetic (▲), propionic (●), citraconic (◊), succinic (□) anhydride and of the non-modified enzyme (×) in 60% aqueous ethanol (A), acetonitrile (B) or 1,4-dioxane (C) at 25 °C. Standard deviations were within 5% of the experimental values. Enzyme concentration: 0.04 mg/ml. The enzyme activities measured at 0 min (acetyl 1.13 U/ml, propionyl 0.93 U/ml, citraconyl 0.84 U/ml, succinyl 0.83 U/ml and non-modified 1.05 U/ml) were taken as 100%.

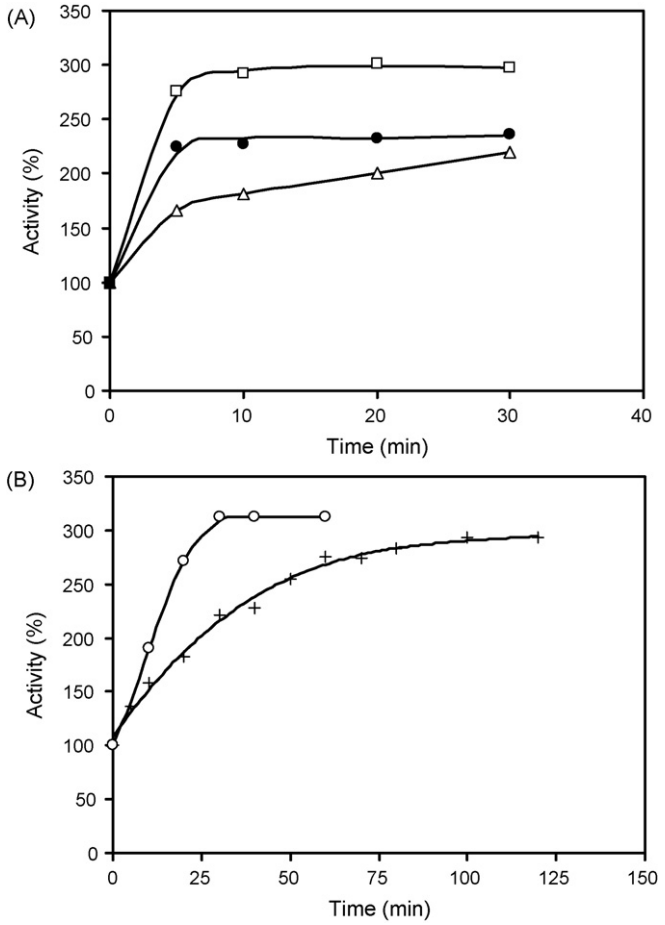

Fig. 3. (A) Activation of α-chymotrypsin modified with phthalic anhydride in 60% aqueous solutions of different organic solvents at 25 °C. Acetonitrile (□), 1,4-dioxane (●) and ethanol (△). (B) In 0.01 M KCl at 40 °C (+) and in the presence of 3 M urea in 0.01 M KCl (○) at 25 °C. Enzyme concentration: 0.04 mg/ml. Enzyme activity: 0.78 U/ml.

The activities of native α-chymotrypsin and of the form modified with phthalic anhydride and activated in 60% ethanol and 0.01 M KCl (60 min, 40 °C) are compared at different ethanol concentrations in Fig. 4. The activated enzyme preserved 68% of its activity even at 80% ethanol, while the native enzyme had practically lost its activity in 60% ethanol after incubation for 20 min at 25 °C. The stability of the activated enzyme modified with phthalic anhydride in 60% ethanol at 25 °C is illustrated in Fig. 5. The unmodified enzyme had lost its activity after incubation for 20 min, whereas that modified with phthalic anhydride preserved about 70% after incubation for 2 h. High stability was measured for the activated modified enzyme form (80% after a 2-h incubation at 25 °C) in 60% acetonitrile and 1,4-dioxane, while the non-modified enzyme lost its catalytic activity within 10 min in 1,4-dioxane and 60 min in acetonitrile (Fig. 6).

In order to establish whether the modifications caused any change in the tertiary structure of α-chymotrypsin, CD measurements were performed in the near-UV region (250–300 nm). The CD spectrum of α-chymotrypsin in this region is a composite of disulfide $n\sigma^*$, tyrosyl L_b and tryptophanyl L_a and L_b contributions. The L_b transition is extremely sensitive, but the L_a transition is less sensitive to conformational changes [16,17]. Since the contributions

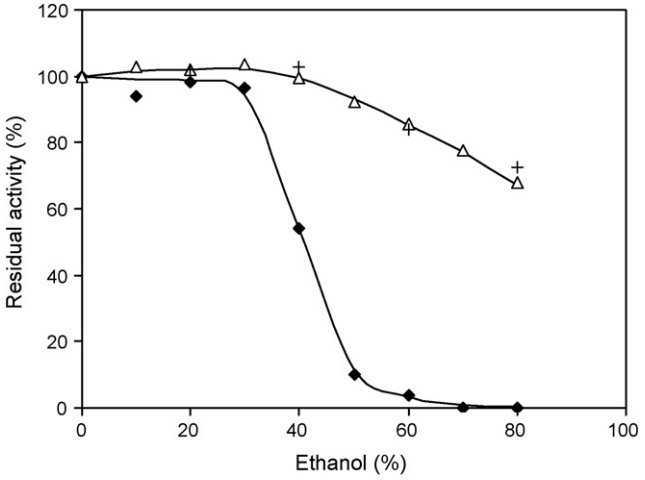

Fig. 4. Effects of different concentrations of aqueous ethanol on the activities of α-chymotrypsin modified with phthalic anhydride activated in 30% aqueous ethanol (△), and after incubation in 0.01 M KCl for 60 min at 40 °C (+) and of non-modified α-chymotrypsin after incubation for 20 min at 25 °C (♦). Enzyme concentration: 0.04 mg/ml. Activities of activated enzymes: 1.45 and 1.96 U/ml, and of non-modified enzyme: 1.2 U/ml.

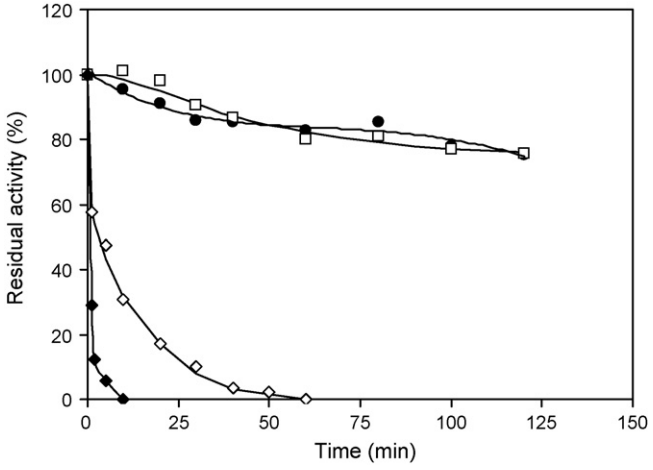

Fig. 6. Stabilities of α-chymotrypsin modified with phthalic anhydride at 25 °C after incubation for 30 min in 60% aqueous acetonitrile (□) or 60% aqueous 1,4-dioxane (●) at 25 °C. Native enzyme in 60% aqueous 1,4-dioxane (♦), and in 60% acetonitrile (◊). Activities: non-modified enzyme: 1.2 U/ml, and activated enzymes: 2.12 and 1.66 U/ml.

of the aromatic groups predominate in the near-UV region, the spectrum is sensitive to the distance between them, and in turn to the compactness of the protein. The intensity is enhanced when the aromatic residues come into closer contact with each other.

The near-UV CD spectra in water and in 60% ethanol of α-chymotrypsin and its versions modified with propionic, succinic and phthalic anhydride are presented in Figs. 7 and 8. The non-modified enzyme gives a negative CD spectrum in water (pH ∼ 7), but changes in sign and intensity are observed in aqueous ethanol, reflecting the formation of a more compact structure. α-Chymotrypsin is an all-β-protein, with distorted or short irregular strands, which may cause the negative far-UV CD band to shift from the ideal β-sheet position of ∼216 nm to lower wavelengths in water [18]. In an earlier paper, we demonstrated that in 60% aqueous ethanol, near neutral pH, a conformational shift occurs and the enzyme adopts a typical β-sheet conformation allowing the enzyme to form a more compact structure [19].

In water, the spectra of propionic, succinic and phthalic anhydride-modified forms of the enzyme are similar to that of

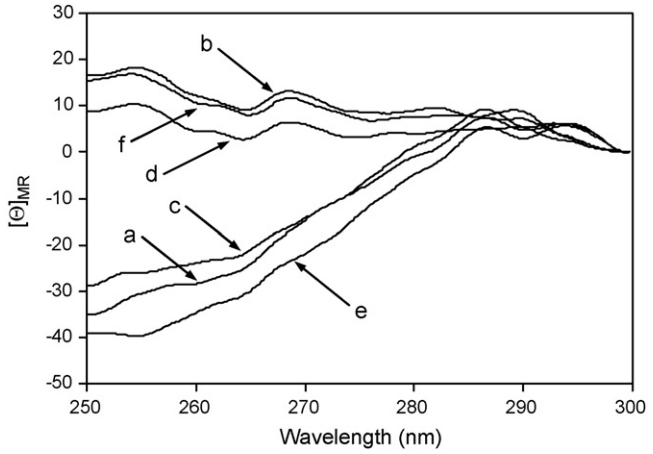

Fig. 7. CD spectra of non-modified and modified α-chymotrypsin forms in water and in 60% ethanol (pH ∼ 7). Non-modified enzyme in water (a) and in 60% ethanol (b); propionylated enzyme in water (c) and in 60% ethanol (d); succinylated enzyme in water (e) and in 60% ethanol (f).

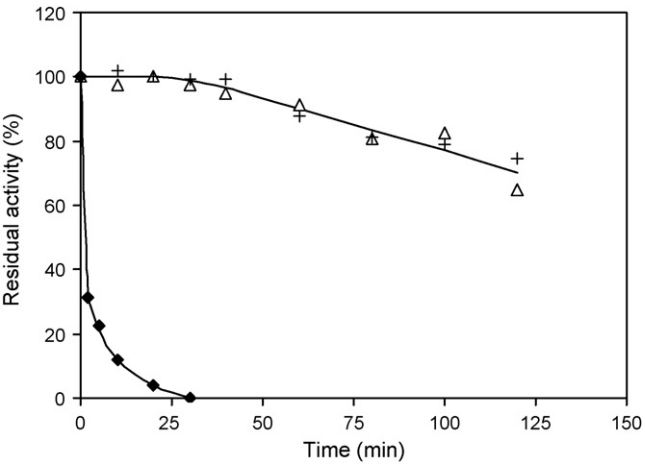

Fig. 5. Stabilities of unmodified α-chymotrypsin (♦) and α-chymotrypsin modified with phthalic anhydride at 25 °C in 60% ethanol (△), after incubation for 30 min at 25 °C and incubation in 0.01 M KCl for 60 min at 40 °C (+). Activities: non-modified enzyme 1.2 U/ml, and activated enzymes: 1.72 and 2.03 U/ml.

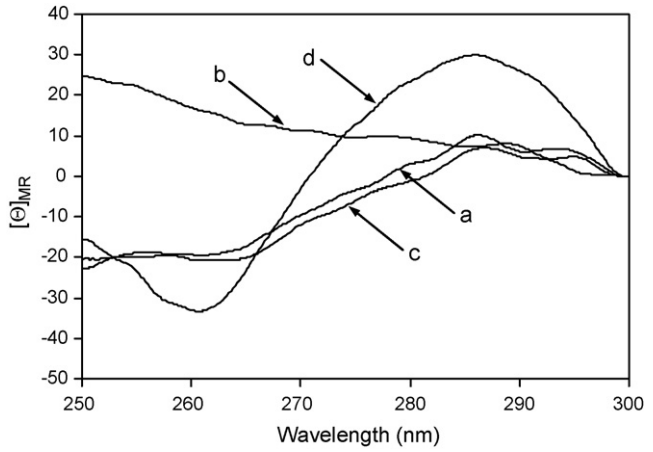

Fig. 8. CD spectra of non-modified α-chymotrypsin in water (a) and in 60% aqueous ethanol (pH ∼ 7) (b). Enzyme modified with phthalic anhydride in water (c) and in 60% aqueous ethanol (d).

unmodified enzyme, though with some variation in their intensities (Figs. 7 and 8). In 60% ethanol, the succinylated and propionylated forms of the enzyme demonstrate small increases in the band intensities (Fig. 7), whereas much more profound spectral changes are to be seen for the form modified with phthalic anhydride (Fig. 8). Two distinct bands appear: a positive contribution with a maximum at 286 nm, which may be due to the increased interaction between the aromatic chromophores, and a negative contribution at ~258 nm, possibly reflecting changes in disulfide contribution. In acetonitrile, the same spectral changes were detected for the phthalic anhydride-modified enzyme (data not shown). These spectral changes may be brought about by the introduction of an additional aromatic group on a lysine side-chain (or the N-terminal amino group) which (due to the changes in the secondary structure in ethanol [19]) comes into closer contact with other aromatic groups. Neither the decreased positive charges in the propionylated form nor the increased negative charges in the succinylated form cause significant conformational changes relative to the situations observed for the non-modified enzyme in water and in aqueous ethanol.

4. Conclusions

Modification of the activity of an enzyme through alteration of the side-chains of functionally important amino acids is an important technique in enzyme technology. The reactions of primary amino groups of α-chymotrypsin with anhydrides result in changes in the enzyme activity and stability. The introduction of acetyl, propionyl, succinyl and citraconyl groups did not cause highly significant alterations in the structure of the enzyme, whereas the alterations in the surface charges did result in a greater organic solvent tolerance, especially at higher solvent concentrations (Fig. 1). The form of the enzyme modified with phthalic anhydride, however, exhibited much higher stability in the studied aqueous organic solvents than the enzyme forms modified with aliphatic acid anhydrides. The presence of an additional aromatic ring and a negative charge caused dramatic changes in both activity and stability (Fig. 3A and B) and also in the tertiary structure of α-chymotrypsin (Fig. 7). The reorientation of the aromatic side-chains of the enzyme is probably responsible for the significant activation both in water and in aqueous organic media.

The higher stabilities of modified forms of α-chymotrypsin might be advantageous in applications in organic syntheses. To elucidate the mechanism of activation at a molecular level, further investigations are in progress.

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