Detection of S_1-P_1 and S_3-P_3 Interactions between Papain and Four Synthetic Substrates

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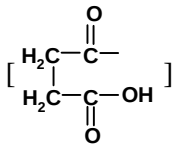
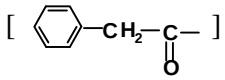
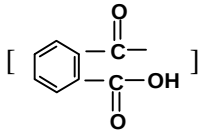
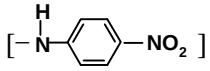
ABSTRACT

In this study, the S_1 - P_1 and S_3 - P_3 interactions between papain and four synthetic peptide substrates were found as to be important. The values of K_m were estimated as to be practically identical between these substrates; this latter is supporting the conclusions obtained by considering the estimated values of other kinetic parameters. Nevertheless, based on the estimated k_{cat} and/or k_{cat}/K_m parameters of the used substrates, we concluded that an aromatic ring at the P_3 position, and a positively charged side chain of the residue at the P_1 position of the synthetic substrates were favored considerably their interaction with papain.

Key words: Papain-Substrates, Enzyme-Substrate Interactions

Abbreviations used

Suc	Succinyl	
Cbz	Benzyloxycarbonyl	
Pht	Phthalyl	
pNA	p-nitroanilide	

INTRODUCTION

It has been reported that the active site of cysteine proteinases (papain - EC 3.4.22.2) comprises seven subsites (Schechter & Berger, 1967). This is well accepted in cases where synthetic peptide substrates are used. Interactions of the S_1' - P_1' and S_2 - P_2 character have been found as the more important ones (Schechter & Berger, 1968; Patel et al., 1992; Kim et al., 1992).

We investigated the S_1 - P_1 and S_3 - P_3 interactions between purified Papain and four

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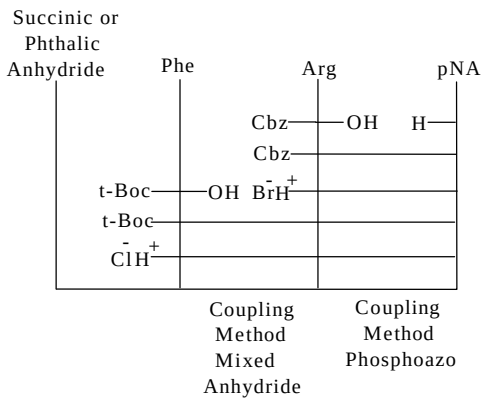
synthetic peptide substrates. Based on their K_m values we cannot discriminated differences between these substrates. However, based on their k_{cat} and/or k_{cat}/K_m we shown that an aromatic ring at the P_3 position and a positively charged side chain of the residue at the P_1 position of the synthetic substrates favored considerably the interactions of theses substrates with Papain.

MATERIALS AND METHODS

All chemicals, including Cbz-Phe-Arg-pNA substrate, were of analytical grade and purchased from Sigma. Papain (EC 3.4.22.2) was further purified by affinity column as described previously (Blumberg et al., 1970) and migrated as a single band of M_r=25000 on SDS/PAGE (Fairbanks et al., 1971); it was active-site titrated with E-64 (Barrett et al., 1982) and found more than 75% active.

The synthesis of two substrates having the general formula **Y-Phe-Leu-pNA**, where Y = {Suc-, Pht-}, has been described elsewhere (Papamichael et al., 1999). The substrate Suc-Phe-Arg-pNA was synthesized from t-BOC-Phe, Cbz-Arg and the appropriate chromophore by using both the mixed anhydride (Greenstein & Winitz, 1961) and phosphoazo methods (Oyamada et al., 1991) according to **Scheme I**. The incorporation of the Suc group was performed as described previously (Bieth et al., 1974). The substrate was purified by reversed phase HPLC (Sephasil Peptide Pharmacia C_{18} column), and its purity was checked by TLC; its structure was assigned by ^{1}H-N.M.R. spectrometry (Brucker AMX-400 MHz).

Scheme I



Initial velocities of enzymatic reactions were measured spectrophotometrically at 410 nm (ε_{pNA}=8800 $M^{-1}cm^{-1}$). In this work were used a Perkin Elmer L15 double beam spectrophotometer. In all cases a typical kinetic run was performed at 25^{o}C as described previously (Tchoupé et al., 1991). The total content of DMSO was kept always constant at 5% (^{v}/v). Each singular kinetic measurement was repeated eight times. From these measurements we estimated the parameters K_m, k_{cat} and k_{cat}/K_m for all used substrates.

The least-squares criterion of convergence has been used throughout in this work. In most cases, robust weighting was also applied to omit observations the errors of which are exceeding the error range of other observations (Chatterjee & Price, 1977).

RESULTS AND DISCUSSION

Table 1: The estimated values of Michaelis-Menten kinetic parameters, of the used substrates

Substrate	Kinetic Parameters
Cbz-Phe-Arg-pNA	K_m = 0.26 mM k_{cat} = 31.42 s^{-1} $\frac{k_{cat}}{K_m}$ =120.85 mM^{-1} s^{-1}
Suc-Phe-Arg-pNA	K_m = 0.26 mM k_{cat} = 6.72 s^{-1} $\frac{k_{cat}}{K_m}$ = 25.85 mM^{-1} s^{-1}
Suc-Phe-Leu-pNA	K_m = 0.47 mM k_{cat} = 1.51 s^{-1} $\frac{k_{cat}}{K_m}$ = 3.21 mM^{-1} s^{-1}
Pht-Phe-Leu-pNA	K_m = 0.26 mM k_{cat} = 0.10 s^{-1} $\frac{k_{cat}}{K_m}$ = 0.39 mM^{-1} s^{-1}

Kinetic measurements were performed using the four following substrates: Cbz-Phe-Arg-pNA, Suc-Phe-Arg-pNA, Suc-Phe-Leu-pNA, and Pht-Phe-Leu-pNA. The results from these measurements are appeared in Table 1.To avoid overcrowding of the Table 1, the errors on the parameters are not given. In all cases standard errors were less than 5%.

In all cases, the Michaelis - Menten equation was best fitted the experimental data from the kinetic measurements. The goodness-of-fit index was found practically equal to unity, and all kinetic parameters (Table 1) were estimated for a 95% confidence interval (UltraFit, 1991).

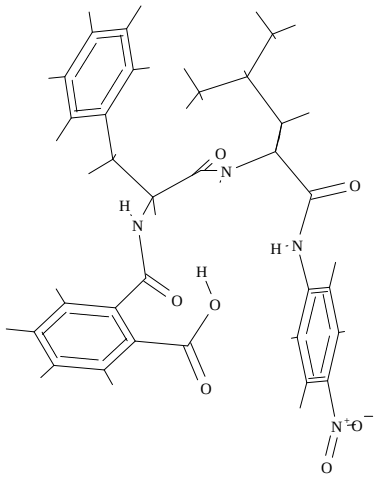
By taking into account the estimated kinetic parameters we can conclude that:

(a) **By comparing K_m:** The values of this parameter were estimated almost equal between all four used substrates. Therefore, all these substrates exhibit equal affinities to Papain. This result was helpful in comparing the used substrates based on the rest two Michaelis parameters.

(b) **By comparing k_{cat}:** The differences between Cbz-Phe-Arg-pNA and Suc-Phe-Arg-pNA, as well as between Suc-Phe-Leu-pNA and Pht-Phe-Leu-pNA, are shown the importance of the S_3 - P_3 interactions. These differences propose that an aromatic ring (Cbz) is preferred by Papain, instead of a charged group (Suc). An objection on this latter statement could be raised by considering the very low estimated value of k_{cat}, for the Pht-Phe-Leu-pNA substrate, as compared to that of Suc-Phe-Leu-pNA. This disagreement is based, most probably, on a hydrogen bond which is likely to exist between the carboxyl proton of Phthalic acid and the carboxylic oxygen of the amide bond between Phthalic acid and α-amino group of the Phe-residue. In **Scheme II**, it is presented the structure of Pht-Phe-Leu-pNA substrate. This structure was calculated by geometric minimization using MM2 parameters. Similarly, regarding the S_1 - P_1 interactions, a positively charged group like that of the side chain of Arg-residue is preferred instead of an aliphatic side chain as it is that of Leu-residue. However, this latter seems to be of less importance than that of the S_3 - P_3 interactions.

(c) **By comparing k_{cat}/K_m:** Similar conclusion, as comparing by k_{cat}, can be withdrawn by taking into account this kinetic parameter though in a more pronounced way.

Scheme II



RESUMO

Neste estudo, o S_1 - P_1 e S_3 - P_3, interações entre papaina e quatro substratos sintéticos de pepetídios foram considerados importantes. Os valores de K_m foram estimados e são praticamente idênticos entre esses substratos; Isso dá suporte as conclusões obtidas, considerando os valores parâmetros cinéticos estimados. No obstante, baseou na estimação parâmetros kcat e/ou k_{cat} /K_m dos substratos utilizados. Se pode concluir que um anel aromático na posição P_3, e uma corrente carregada positivamente da cadeia do resíduo na posição P_1 dos substratos sintéticos favoreceram interação com a papaina.

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