COMPARATIVE KINETIC STUDIES ON PAPAIN AND SUCCINYLPAPAIN WITH DIFFERENT PROTEIN SUBSTRATES

Hans Gerhard LÖFFLER and Friedhelm SCHNEIDER
Physiologisch-chemisches Institut II der Universität Marburg, Marburg/Lahn, Germany

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1. Introduction

In a previous communication we have described the preparation and some kinetic and chemical properties of succinylpapain [1]. The most striking differences in the physical properties between papain and succinylpapain were a considerable enhancement of solubility of papain by succinylation and a difference of the isoelectric points being nearly 4.5 pH units [2]. Papain and succinylpapain are therefore very attractive for studying the importance of the surface charge of an enzyme for the interaction between enzyme and substrate molecules, especially since the catalytic activity of papain is scarcely impaired by succinylation. In the present paper we describe our experiments on the determination of Michaelis-Menten parameters for the hydrolysis by papain and succinylpapain of protein substrates with different properties.

2. Materials and methods

Papain (spec. activ. 30 U/mg) was a gift of Boehringer, Mannheim. Succinylpapain was prepared as previously described [1,2]. Casein, protaminsulfat, trinitrobenzenesulfonic acid were purchased from Serva, Heidelberg. Gelatin, benzoyl-L-argininethylester and buffer substances were from Merck, Darmstadt.

Casein was used without further purification. The free protein base was prepared from protaminsulfat by treatment of the protein with Dowex 2 X 10, 200–400 mesh followed by lyophilisation. Dimethylgelatine and dimethylcasein were prepared as described by Lin et al. [3].

Activity measurements were performed by the micro protease test according to Lin et al. [3] using trinitrobenzenesulfonic acid for the determination of amino groups formed during proteolysis. The activity with benzoyl-L-argininethylester was measured with a pH-stat arrangement from Radiometer, Copenhagen.

3. Results and discussion

Kinetic parameters of papain and succinylpapain with benzoyl-L-argininethylester and the relative activities with casein and protamin are compared in table 1. In spite of some essential changes in the physical properties of the enzyme by succinylation no decrease in activity is observed.

In order to get further information on the effect of succinylation of papain on the interaction with and the binding of macromolecular substrates we have tried to determine kinetic parameters for the hydrolysis by papain and succinylpapain of proteins with different mol. wts and isoelectric points.

Three proteins were chosen as substrates for these investigations: the basic protamin, the acidic protein dimethylcasein and the nearly neutral dimethylgelatin. Since (according to the amino acid analysis) protamin was free of lysine a dimethylation of amino groups was unnecessary. A quantitative dimethylation of the lysin residues of casein was accomplished without difficulties, while only 50% of the lysin amino groups of gelatine could be dimethylated even under drastic conditions.

The number of amino groups formed by proteolysis is plotted against time in fig. 1; the initial rates were taken from these diagrams. Plotting $[\frac{E}{v}]$ against $\frac{1}{[S]}$

Table 1
Kinetic parameters and activity of papain and succinylpapain

	Isoelectric point [5]	K_m app* [mol/l]	k_{cat}* [min^{-1}]	% activity** with casein (anionic)	% activity*** with protamin (kationic)
Papain	9.6	1.8×10^{-2}	485	100	100
Succinyl-papain	4.3	1.2×10^{-2}	505	120 ± 10	200 ± 20

* Substrate N-benzoyl-L-argininethylester, pH 6.5, 25°C
** Conditions 1 mg/ml, pH 8.0 (Kunitz test) [4]
*** Conditions 5 mg/ml, pH 8.2 (pH-stat test)

one gets Dalziel-diagrams shown in fig. 4 a–c; k_{cat} and K_m may be taken directly from these graphs; these values are summarized in table 2.

The following conclusions may be drawn from our experiments: The determination of initial rates of proteolysis by measuring the liberation of amino groups using trinitrobenzenesulfonic acid opens the possibility for the evaluation of Michaelis-Menten parameters for macromolecular substrates. However complex these parameters may be, one can study the influence of the surface charge of the enzyme on these constants and the results may give some valuable information

Table 2
Kinetic parameters of the proteolysis by papain and succinylpapain of different protein substrates

	K_m [mg/ml]		k_{cat} [min^{-1}]	
	Papain	Succinyl-papain	Papain	Succinyl-papain
Protamine	6.6	1.5	2×10^3	2×10^3
Dimethyl-gelatin	0.7	0.4	6.1×10^2	7.6×10^2
Dimethyl-casein	0.1	1.0	2.5×10^2	1.2×10^3

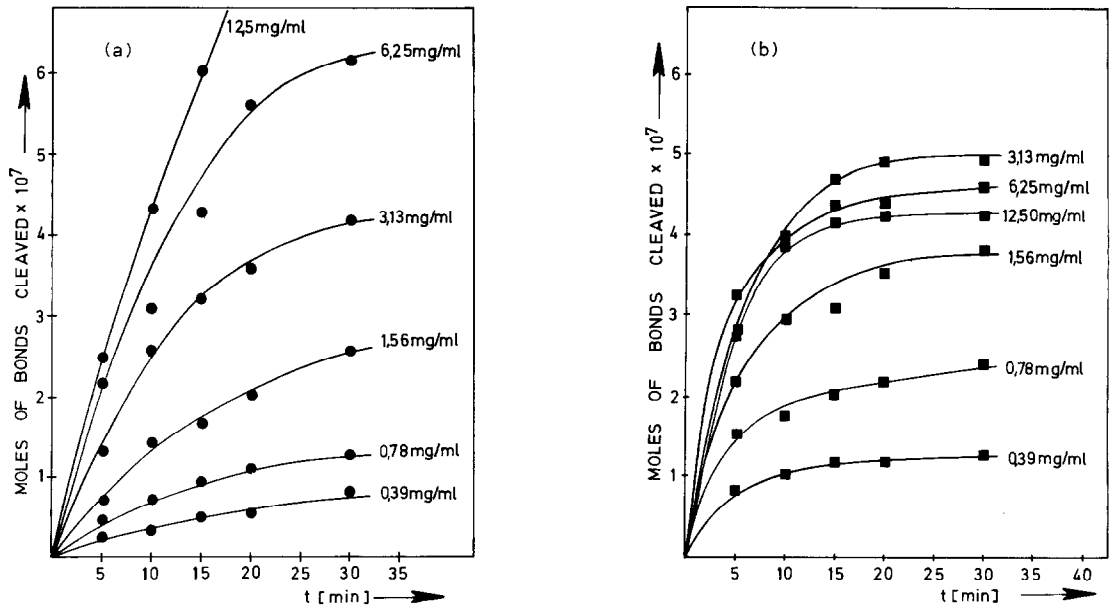


Fig. 1. Time dependence of the liberation of amino groups of protamin during proteolysis by (a) papain and (b) succinylpapain. Conditions: 0.1 M phosphate buffer, pH 7.5, enzyme concentration 4.3×10^{-8} M. Determination of amino groups according to [3]

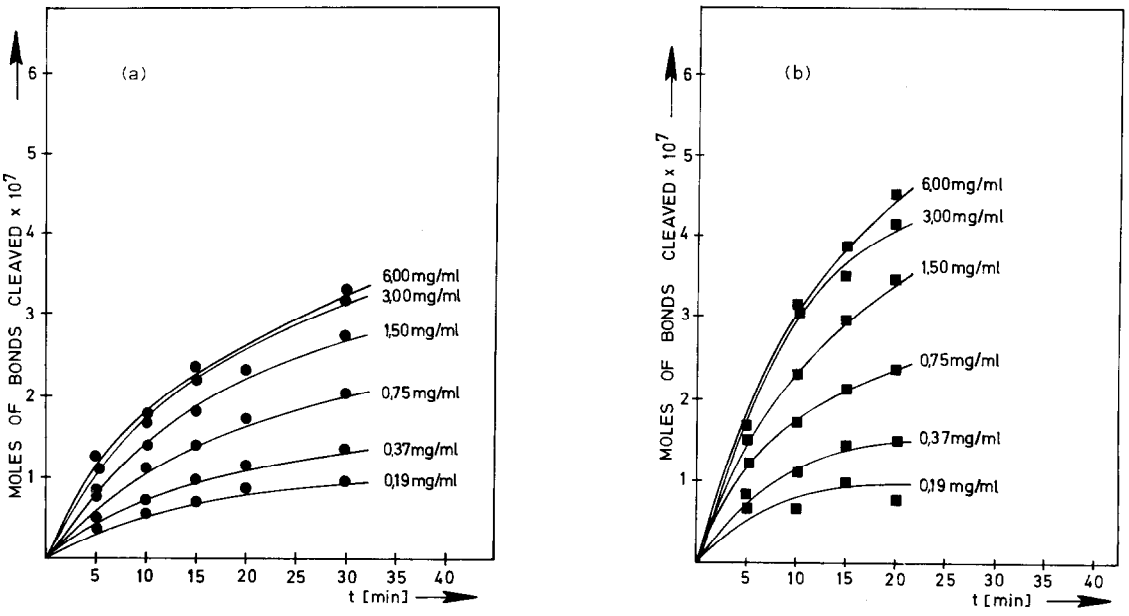


Fig. 2. Time dependence of the liberation of amino groups of dimethylgelatine during proteolysis by (a) papain and (b) succinylpapain. Conditions: 0.1 M phosphate buffer, pH 7.5 enzyme concentration 4.3×10^{-8} M. Determination of amino groups according to [3].

on the interaction of enzymes with macromolecular substrates.

Comparing the hydrolysis of the positive charged substrate protamin by the positive charged papain and by the negative charged succinylpapain (fig. 1) one recognizes that at high substrate concentrations the initial rate of the liberation of amino groups is about 50% higher with succinylpapain than with papain. This rate enhancement is a consequence of an increase of the affinity of papain for protamin by succinylation since k_{cat} is not affected. The proteolysis by succinylpapain stops however after 20 min as a result of an apparent 'product inhibition'. This product inhibition is more pronounced with succinylpapain than with papain. Therefore the number of peptide bonds cleaved by succinylpapain finally is lower than by papain. At low and medium substrate concentrations we find a higher degree of proteolysis by succinylpapain than by papain. In this case the greater 'affinity' (lower K_m) of succinylpapain for protamin determines the rate and degree of liberation of amino groups, the effect of 'product inhibition' being repressed. The simplest idea on the enhancement of the affinity of succinylpapain for protamin is the electrostatic attraction of the reaction partners. This electrostatic interaction may not only be the reason for the decrease of the Michaelis constant but also for the strong 'product inhibition' of succinylpapain especially at high substrate concentrations.

Exactly the reverse behavior is observed with the negative charged substrate dimethylcasein (fig. 2). The effects are some less pronounced because of the much lower substrate concentration, which must be utilized (due to substrate inhibition, see below).

In this case the affinity of the positive charged papain for the negative charged dimethylcasein is ten times greater than that of the negative charged succinylpapain. 'Product inhibition' is therefore particularly marked for the hydrolysis of dimethylcasein by papain. Generally it seems as if 'product inhibition' is the greater the lower the K_m value of the respective substrate.

The Dalziel-plot with dimethylcasein shows a 'substrate inhibition' (fig. 4c). Papain and succinylpapain are already inhibited by relative low dimethylcasein concentrations. Lin et al. [3] have also observed

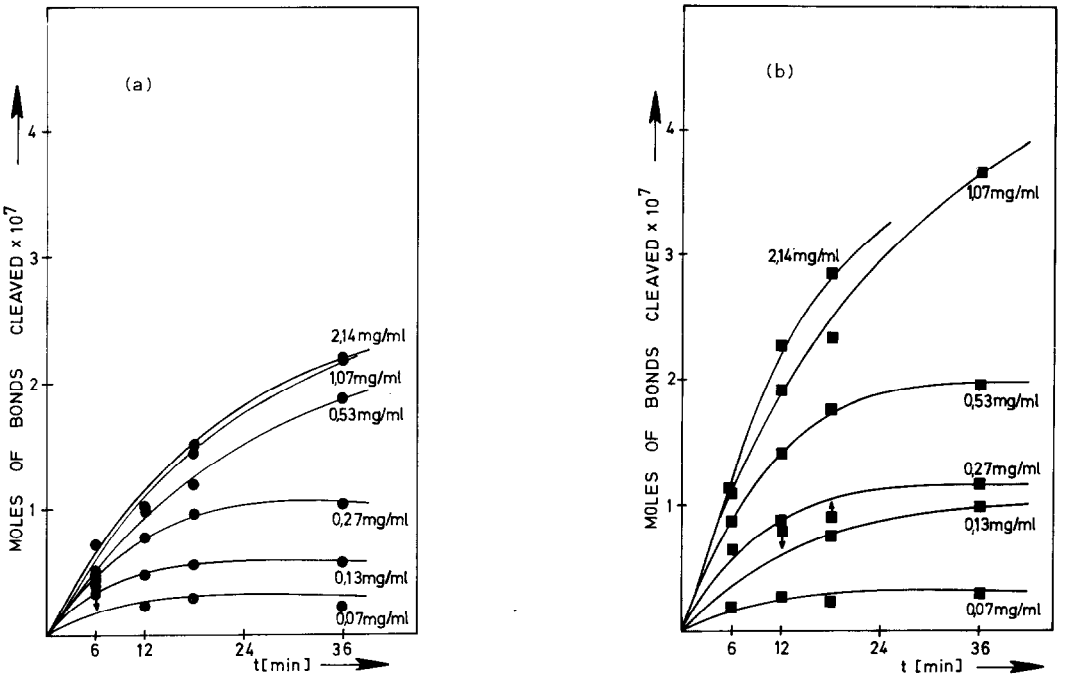
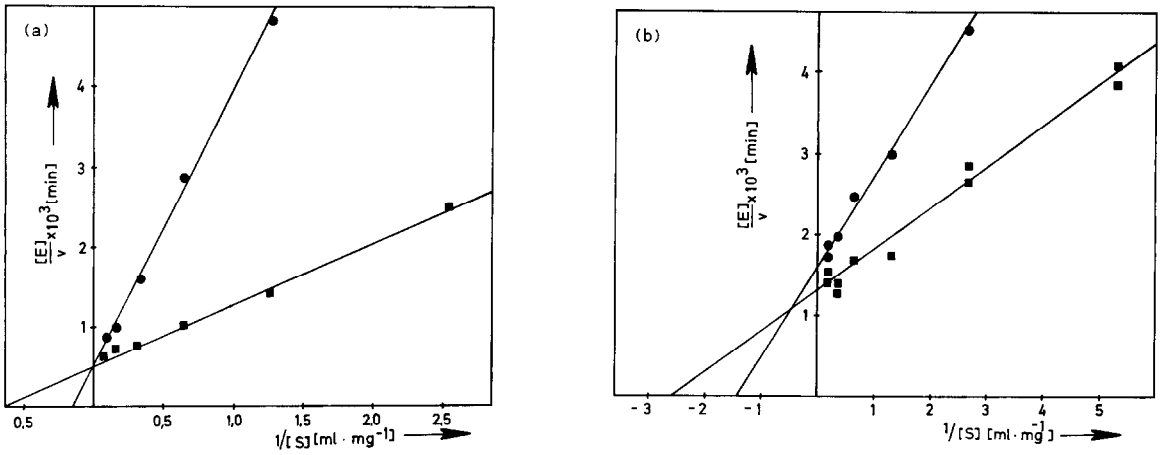


Fig. 3. Time dependence of the liberation of amino groups of dimethylcasein during proteolysis by (a) papain and (b) succinyl-papain. Conditions: 0.1 M phosphate buffer, pH 7.5, enzyme concentration 4.3 X 10^{-8} M. Deterination of amino groups according to [3].

'substrate inhibition' of trypsin by casein. The deviation of the Dalziel-plot from linearity may be produced by a decrease in the effective concentration of the substrate as a result of aggregation of dimethylcasein above a certain concentration and not by an ineffective binding of the substrate.

The liberation of amino groups from protamine by papain (fig. 1a) and from dimethylcasein by succinyl-papain (fig. 2b) is a linear function of time only with high substrate concentrations. With medium and low concentration the plots are non-linear. The deviation from linearity with medium and low substrate



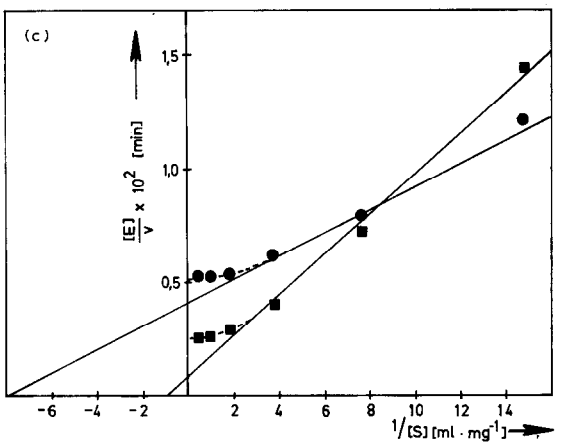


Fig. 4. Dalziel-diagram ($\frac{[E]}{v}$ against 1/[S]) for the hydrolysis of a) *Protamin* by papain (●—●—●) and succinylpapain (■—■—■) b) for dimethylgelatin c) for dimethylcasein.

concentration is the consequence of a rapid decrease in the number of cleavable bonds and of the apparent 'product inhibition'. This 'product inhibition' results from an unspecific binding of positive charged peptide fragments by the negative charged enzyme and vice versa. It is unlikely that the peptide fragments are bound at the active center. Otherwise the inhibition should be equally strong with papain and succinylpapain since the active center of the enzyme is obviously not impaired by succinylation. With nearly all of the substrates tested the k_{cat} values of succinylpapain are greater than those of papain (table 2). This is a somehow unexpected result and all ideas on the reason of the enhancement of the catalytic activity of papain by succinylation are rather speculative. One must realise however that k_{cat} is most probably a complex constant, which is not only a parameter for the catalytic potency of the enzyme.

The nearly neutral protein dimethylgelatine is a little better substrat for succinylpapain than for papain (table 2). The initial rate of the formation of amino groups as well as the degree of proteolysis is greater with succinylpapain than with papain (fig. 3). Comparable investigations on the kinetics of proteolysis have been published by Gertler [5] and Lin et al. [3]. However neither determined initial rates, they quenched proteolysis at different substrate concentrations, always at the same time.

References

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