BBA 66522

THE EFFECT OF SALTS UPON THE pH DEPENDENCE OF THE ACTIVITY OF PAPAIN AND SUCCINYL-PAPAIN

L. A. AE. SLUYTERMAN AND M. J. M. DE GRAAF
Philips Research Laboratories, N.V. Philips' Gloeilampenfabrieken, Eindhoven (The Netherlands)

(Received October 25th, 1971)

SUMMARY

Papain (EC 3.4.4.10) and succinyl-papain exhibit isoelectric points of 9.6 and 4.3, respectively.

The acid limb of the pH activity curve in the pH range of 3.5–4.5 of native papain and the basic limb in the pH range of 8–9 of succinyl-papain are shifted by monovalent salts, whereas the basic limb of native papain and the acid limb of succinyl-papain are not shifted by KCl. The K_m and not the k_{cat} with benzoylglycine ethyl ester as substrate is affected by salt. The order of the effectiveness of the ions is $I^- > NO_3^- > Br^- > Cl^-$ and $Li^+ > K^+$. These effects can be explained in terms of non-specific binding of cations and anions and electrostatic interactions.

INTRODUCTION

STOCKELL AND SMITH[1] examined the effect of KCl upon the activity of papain (EC 3.4.4.10), at pH 5.2–5.8, on benzoyl-arginine amide. They observed a small increase at low KCl concentrations (up to 0.05 M KCl) and no change in activity between 0.05 and 0.4 M KCl. Therefore 0.3 M KCl was used as a standard medium for kinetic studies[2].

WOLTHERS[3] observed that KCl inhibited the activity at low pH, the inhibitory action increasing with decreasing pH. Because the inhibitory action on the hydrolysis of benzoyl-arginine ethyl ester and benzoyl-glycine ethyl ester was found to be due to an increase in K_m, and because plots of $1/v$ *versus* salt concentration proved to be linear, the preferred explanation was a direct competitive inhibition by the anion, governed by the carboxyl group of Asp 158 at the entrance to the trough of the active site.

The measurements of WOLTHERS[3] have now been repeated in a systematic manner and have been extended to the basic limb of the pH-activity curve and to succinylated papain.

EXPERIMENTAL

Mercuric papain was prepared by elution from an agarose mercurial column[4].

The KCl and dimethylsulphoxide of the resulting papain solution were removed by dialysis in an ultrafiltration cell (Amicon Ltd, U.K., membrane UM 10).

If most of the dimethylsulphoxide was not removed prior to succinylation, some denaturation occurred during this reaction.

Succinylation

The method of HABEEB *et al.*[5] was slightly modified. To a 20 ml suspension of 1% mercuric papain in 0.1 M pyrophosphate buffer of pH 8.0, 480 mg solid succinic anhydride (Merck) was added in small portions at an ambient temperature of 23°. Soon after the reaction had started, the suspension clarified. The pH was maintained within the limits of ±0.1 pH units by addition of 1 M NaOH and was allowed to drop to about pH 6.5 after the addition of the last crystals of anhydride. The solution was dialyzed by ultrafiltration. Assay at pH 6.0 of benzoyl-glycine ethyl ester hydrolysis demonstrated a loss of activity of only 10%.

Ninhydrin reaction

The method was mainly that recommended by MOORE[6]. To a solution of 4 g ninhydrin and 1 g hydrindantin (both from Fluka, Switzerland) in 150 ml dimethylsulphoxide, 50 ml 0.4 M acetate buffer (pH 5.0) was added in an atmosphere of nitrogen. The mixture was stored at 4°. Of this mixture, 2 ml were added to 0.2 ml protein solution containing up to 0.7 μmole amino nitrogen, heated in boiling water for 15 min, and cooled. After addition of 5 ml 50% propanol the absorbance was read at 570 nm. In succinyl-papain 68–70% of the amino groups proved to be acylated.

Amino nitrogen

The combination of the nitrous acid reaction and of gas chromatographic analysis of the resulting gases, recommended by BELEN'KII AND ORESTOVA[7], proved to be a convenient method, especially after a few modifications had been introduced. In the reaction vessel the funnel, syringe, and flask were placed in a plane perpendicular to the axis of the stopcock. The single bore in the plug of the stopcock was such that funnel and syringe or syringe and flask could be interconnected. During the assay the nitrous acid in the bore was replaced by air-free water. Between assays the flask was not disconnected from the helium inlet and was washed with air-free water from the syringe.

Results: papain; calculated, 11.0 amino groups; found, 11.6 groups; succinyl-papain, 3.0 groups.

Hydroxamate reaction

The degree of esterification of the hydroxyl groups was determined according to the method of PILZ[8], with succinic monomethyl ester as a standard. 3.8–4.0 ester groups were found in succinyl-papain.

Only a little transient acylation of tyrosines was observed by absorption in the 250–300 nm range.

Activity

Activity with benzoyl-glycine ethyl ester was determined by automatic titration at 25° using Radiometer equipment (Copenhagen), consisting of titrator TTT1,

titrigraph SBR 2, and automatic burette ABU 12 (burette of 0.25 ml containing 0.1 M NaOH).

For the determination of activity dependence on pH the reaction mixture contained 2.5 mM benzoyl-glycine ethyl ester, 1 mM EDTA and 2.5 mM dithiothreitol (Calbiochem) up to pH 8.0 or 1 mM dithiothreitol at pH >8.0.

For the determination of the dependence of activity on salt concentration at pH 3.9 and at pH 8.7 the substrate concentration was somewhat increased: 5 mM at pH 3.9 and 4 mM at 8.7. Furthermore 1 mM EDTA, 2.5 mM dithiothreitol at pH 3.9 or 1 mM at pH 8.7 were added with the required amount of salt.

For the determination of activity dependence on substrate concentration at pH 4.0 and at pH 8.5 the substrate concentration was 4, 8, 12, 16, 20 and 32 mM, the other conditions remaining the same as above. The results were plotted as $1/v$ *versus* $1/S$. Straight lines were drawn according to the method of least squares, calculated with a computer.

Appropriate corrections to the reaction rates were applied, in the acid range for incomplete ionization of the product benzoyl-glycine (pK taken as 3.6, in accordance with ref. 9) and in the basic range for spontaneous hydrolysis of the ester. In all cases the initial rates were measured.

Electrofocusing

An analytical column of 110 ml (LKB, Sweden) was employed.

For native papain, 2% ampholine (pH 8–10) was used. The protein was dissolved in the light solution. The dense (sucrose) cathode solution contained ethanolamine, the light anode solution contained phosphoric acid, in accordance with ref. 10.

Succinyl-papain was run in ampholine (pH 3–6). The protein was dissolved in the dense solution. The dense anode solution contained phosphoric acid, the light cathode solution ethanolamine.

All solvents contained 10% dimethylsulphoxide to prevent isoelectric papain from precipitating. All runs were carried out at 20° for 70 h. The maximum voltage was 600 V. pH values were read on a digital pH meter (Philips, type PW 9408); absorbance was measured at 280 nm in a Unicam SP1800 spectrophotometer.

RESULTS

Isoelectric point

After electrofocusing, native mercuric papain exhibited a double isoelectric point (pI) at pH 9.55 and 9.75, the former peak being the higher one (Fig. 1a, ●—●). Addition of 0.1 M $HgCl_2$ to the ampholine buffer decreased the former and increased the latter peak (Fig. 1a, – – –).

These data suggest that the peak at pH 9.75 is mercuric papain with a blocked thiol group, which is in very slow equilibrium with papain having a free thiol group. Since the pK of the thiol group of papain is 8.6 (ref. 11), the group is almost fully ionized at pH 9.6. Its negative charge will therefore decrease the pI, apparently from 9.75 to 9.55. Confirming this interpretation, papain prepared according to the method of KIMMEL ANS SMITH[11] exhibits a single peak of pI 9.6. In the latter case some papain molecules carry a free thiol group and some a thiol group combined with a half-cystine residue[11]. Both the free thiol group and the half-cystine residue carry a net negative

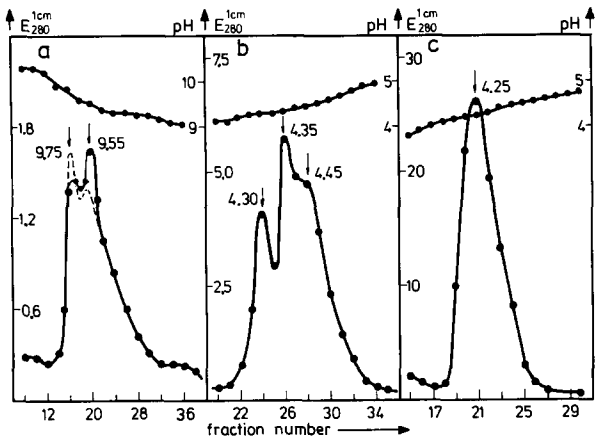

Fig. 1. Electrofocusing of papain and succinyl-papain. (a). Papain. One fraction equals 1.15 ml. (— — —) in the presence of additional mercuric chloride. (b). Partially succinylated papain. One fraction equals 2.0 ml. (c). Succinyl-papain. One fraction equals 2.1 ml.

charge at pH > 9.0 and therefore have an equal effect on the pI of papain.

SMITH *et al.*[12] reported a pI of 8.75, determined by the moving boundary method. The difference between the latter and the present values may be due to the different electrolytes in the medium and to the difference in temperature (1.5° and 20°, respectively).

Papain in which 8 amino groups and 4 hydroxyl groups had been succinylated exhibited a single peak at pH 4.3 (Fig. 1c). Papain which had been less fully succinylated (with half the amount of anhydride) exhibited three peaks. The smallest peak corresponded with the peak of Fig. 1c, those of pI 4.35 and 4.45 were less succinylated papains. Small amounts of the latter material may still be present in the peak of Fig. 1c.

For the present purpose it is sufficient to state that native papain has a pI of 9.6, and papain succinylated as described in the experimental part has a pI of 4.3.

Activity

The overall activity was measured with benzoyl-glycine ethyl ester as an uncharged substrate, at a concentration of 2.5 mM, *i.e.* sufficiently below the K_m values (13 mM at pH 6, ref. 13) to be practically equal to k_{cat}/K_m.

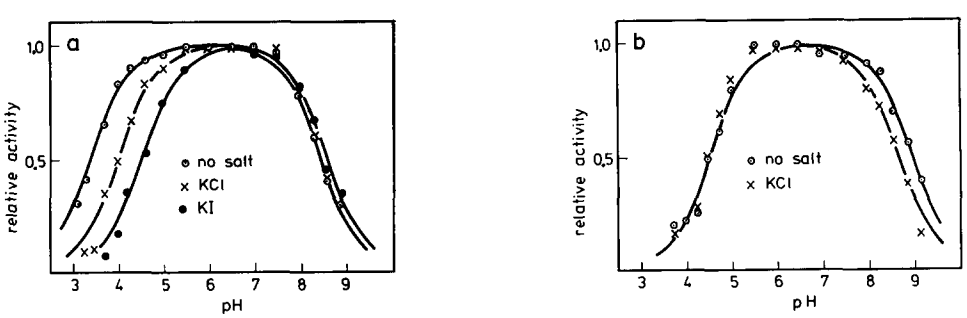

Fig. 2. a. Papain activity as a function of pH. b. Succinyl-papain activity as a function of pH.

TABLE I

APPARENT pK VALUES GOVERNING THE ACTIVITY OF NATIVE PAPAIN (pI 9.6) AND OF SUCCINYL-PAPAIN (pI 4.3)

	Acid limb		*Basic limb*	
	Native	*Succinyl*	*Native*	*Succinyl*
No salt	3.4	4.5	8.4	8.95
0.3 M KCl	4.0	4.5	8.4	8.65
0.3 M KI	4.5	—	8.6	—

At pH 6.0, 0.3 M KCl proved to have a small stimulating effect of 8%, whereas 0.3 M KI had no effect at all.

The effects of pH in the absence and presence of 0.3 M KCl and KI are shown in Fig. 2. The data are presented as values relative to their maximum value. In 0.3 M KCl the acid limb of native papain reaches half its maximum at pH 4.0. However, in the absence of salt the apparent pK is shifted to 3.4. On the other hand, the basic limb of native papain of pK 8.4 is not shifted by KCl. Even more pronounced is the effect of 0.3 M KI: the acid limb is governed by a pK of 4.5, the basic limb is only slightly shifted, to pK 8.6.

With succinyl-papain the opposite occurs: the acid limb is governed by a pK of 4.5 and is not affected by KCl, but the basic pK at 8.65 in 0.3 M KCl is shifted to pK 8.95 in the absence of the salt.

The various effects are summarized in Table I. At a single pH in the acid limb of native papain, at pH 3.9, and at a single pH in the basic limb of succinyl-papain, pH 8.7, the effect of the concentration of a few salts has been examined. The results are plotted as v_0/v_i (v_0, rate in the absence of salt; v_i, rate in the presence of salt) *versus* salt concentration in Fig. 3a and 3b. At pH 3.9 straight lines are obtained, as observed by Wolthers for this and a few other low pH values. The slope of the lines

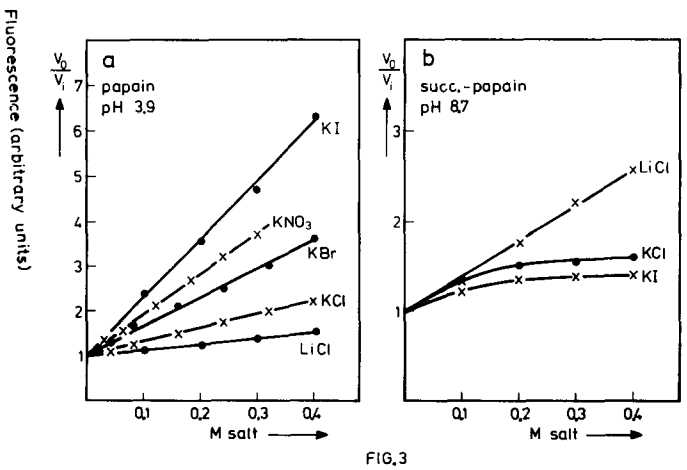

FIG. 3

Fig. 3. a. Papain activity as a function of salt concentration. b. Succinyl-papain activity as a function of salt concentration.

TABLE II

THE EFFECT OF SALTS UPON THE REACTION PARAMETERS OF PAPAIN AND SUCCINYL-PAPAIN

	K_m (mM)	V (relative)
pH 4.0, native papain:		
No salt	13.5 ± 0.5	1.00 ± 0.03
0.3 M KCl	25.0 ± 0.7	0.95 ± 0.03
pH 8.5, succinyl-papain:		
No salt	19.0 ± 1.6	1.00 ± 0.07
0.03 M LiCl	37.0 ± 4.0	1.00 ± 0.09

increases with the following order of anions: $Cl^- < Br^- < NO_3^- < I^-$. The inhibitory effect of LiCl is less than the effect of KCl.

At pH 8.7 with succinyl-papain some of the effects are reversed: LiCl inhibits more strongly than does KCl, whereas KCl inhibits more strongly than KI. Furthermore the effects are much less, and saturation occurs with KCl and KI.

In order to find out whether the K_m or the k_{cat} is affected by salt, the effect of KCl at various substrate concentrations was examined at pH 4.0 with native papain, and that of LiCl at pH 8.5 with succinyl-papain. The results were plotted as double reciprocal plots, after Lineweaver and Burk. Both at pH 4.0 (in accordance with WOLTHERS[3] results) and at pH 8.5, the K_m was affected without significant change in the k_{cat} (see Table II).

DISCUSSION

It is not surprising that succinylation of papain should cause a change in p*I* as large as 5 units, since 4 neutral hydroxyl groups and 8 positive amino groups are converted into negative groups.

The singleness of the peak of succinyl-papain in Fig. 1c, unlike the apparent non-homogeneity of the material of Fig. 1b, indicates that the remaining amino groups in succinyl-papain are rather resistant to succinylation. Examination of the molecular model of papain[14] shows that the majority of the eleven amino groups, including the N-terminal group, are located at the surface of the molecule and point outwards. There are four exceptions. The side chain of Lys 100 lies flat against the surface, and its amino group touches the benzene ring of Tyr 87. This lysine is capable of reacting with phenyl isothiocyanate[15], possibly *via* absorption of the reagent on the nearby tyrosine residue. Lys 39, though at the surface, is sunk slightly below the surrounding surface and forms a hydrogen bond with the carboxyl group of Gly 11. Lys 17 too is not quite free and forms a salt bridge with Glu 50. Lys 174 is buried and hence is quite inaccessible.

All things considered, the lack of reactivity of some of the lysines can be understood from the model, though a more detailed analysis is desirable.

The salt effects can be readily explained by non-specific cation and anion binding and electrostatic interactions. In the absence of added salt the only electrolyte is 1 mM EDTA. At approx. pH 9 dithiothreitol is also partially ionized.

Native papain, owing to its high p*I* of 9.6, will carry a strong net positive charge at pH 3–4 which will tend to repel positive ions such as protons. This repulsion

will lead to a low pK of 3.4 for the group concerned in the activity in the absence of salt.

In the presence of salts the binding of anions will be stronger than the binding of cations, because of the net positive charge of the protein and the intrinsically stronger binding of anions than cations[16,17]. The excess binding of anions will decrease and eventually more than compensate for the initial positive charge of the protein, thus decreasing the repulsion of protons and increasing the pK of the essential group from 3.4 to 4.0 in 0.3 M KCl and to 4.5 in 0.3 M KI. The effectiveness of the anion binding $I^- > NO_3^- > Br^- > Cl^-$ (Fig. 3a) exhibits the same order as observed in the case of gelatin[16]. The ratio of the slopes of the lines for KI and KCl is 4.15. This is quite near to the ratio 3.85 of I^- and Cl^- binding observed with serum albumin[18].

Despite the net positive charge, some binding of cations will also occur, for instance near negative centres, as exemplified by the protons. Such binding partially cancels the effect of the anions. This is evident from the data of KCl and LiCl. Since Li^+ is known to bind more strongly than K^+ (ref. 16), more Li^+ will be bound at a given concentration than K^+. Hence Li^+ will more effectively oppose the effect of the anions than K^+, and therefore the LiCl curve will lie below the KCl curve, as is in fact observed (Fig. 3a).

The basic limb of the activity curve of native papain lies at pH 8–9, *i.e.* not far below the pI. Therefore the net positive charge will be only small and no large salt effect is to be expected; this is, in fact, observed: no effect of KCl, a slight effect of KI.

Since the pI of succinyl-papain is 4.3, the acid limb of the activity curve occurs in the pH range of no net charge. Therefore there is no repulsion of protons, in contrast to what is found with native papain. Hence the pK value of succinyl-papain, 4.5, is higher than the value of 3.4 of native papain (Fig. 3b). The lack of net charge does not lead to a salt shift of the pK value, similarly with the basic limb of native papain.

The basic limb of the activity curve of succinyl-papain is more than 4 units above its pI. Therefore the enzyme carries a strong net negative charge. This charge impedes removal of protons and increases the pK value from 8.4 in native papain to 8.95 in succinyl-papain in the absence of salt. This limb is shifted back to pK 8.65 by 0.3 M KCl, owing to partial charge compensation, this time by stronger cation binding than anion binding. The effect of Li^+, again owing to its better binding ability, is stronger than that of K^+ (Fig. 3b). The cation effect is partially counteracted by anion binding; the binding of I^- is again stronger than binding of Cl^-. Hence KI has a smaller effect than KCl (Fig. 3b).

With proteins, cation binding is less pronounced than anion binding[16,17]. Therefore the salt effects on the negatively charged succinyl-papain are less pronounced (Fig. 3b) than on the positively charged native papain (Fig. 3a). The effects of KCl and KI on succinyl-papain even tend towards a plateau. In the other cases such a plateau is not evident in the salt concentration range examined, indicating more extensive binding.

The observation that salt affects the K_m fits in with previous observations that the k_{cat} of benzoyl-glycine ethyl ester remains constant* in the range of pH 3.6 to 8.7,

* In ref. 9 an increase in k_{cat} has been reported at pH <4. This increase has now been found to be absent if the papain is purified on a column of Sephadex G 75 (Pharmacia, Sweden) or of agarose-mercurial[4].

whereas the K_m increases at both ends of the pH range (Table X of ref. 11). Since salts affect the ionization of the essential groups and the ionization of these groups affect the K_m, salts affect the K_m at fixed pH values in the vicinity of the pK values concerned.

A practical consequence of the present observations is that one should be cautious in interpreting small differences in pK values with different substrates or inhibitors if the salt composition of the medium is not the same.

ACKNOWLEDGEMENT

The authors gratefully acknowledge the discussions with Dr. H. J. van den Berg concerning ion binding in colloid chemistry.

REFERENCES

1 A. STOCKELL AND E. L. SMITH, *J. Biol. Chem.*, 227 (1957) 1.
2 E. L. SMITH AND M. J. PARKER, *J. Biol. Chem.*, 233 (1958) 1387.
3 B. G. WOLTHERS, Thesis, Groningen, 1970.
4 L. A. AE. SLUYTERMAN AND J. WIJDENES, *Biochim. Biophys. Acta*, 200 (1970) 593.
5 A. F. S. A. HABEEB, H. G. CASSIDY AND S. J. SINGER, *Biochim. Biophys. Acta*, 29 (1958) 587.
6 S. MOORE, *J. Biol. Chem.*, 243 (1968) 6281.
7 B. G. BELEN'KII AND V. A. ORESTOVA, *Biokhimiya (Engl. Transl.)*, 30 (1965) 878.
8 W. PILZ, *Z. Anal. Chem.*, 162 (1958) 81.
9 L. A. AE. SLUYTERMAN, *Biochim. Biophys. Acta*, 85 (1964) 305.
10 *Instruction Manual Electrofocusing Equipment*, LKB-Produkter AB Bromma 1, Sweden.
11 A. N. GLAZER AND E. L. SMITH, in P. D. BOYER, *The Enzymes*, 3rd Ed., Vol. 3, Academic Press, New York, 1971, p. 502.
12 E. L. SMITH, J. R. KIMMEL AND D. M. BROWN, *J. Biol. Chem.*, 207 (1954) 533.
13 L. A. AE. SLUYTERMAN, *Biochim. Biophys. Acta*, 151 (1968) 178.
14 J. DRENTH, J. N. JANSONIUS, R. KOEKOEK, H. M. SWEN AND B. G. WOLTHERS, *Nature*, 218 (1968) 929.
15 R. KOEKOEK, Thesis, Groningen, 1969.
16 H. G. BUNGENBERG DE JONG, in H. R. KRUYT, *Colloid Science*, Vol. 2, Elsevier, Amsterdam, 1949, pp. 297–300.
17 J. STEINHARDT AND S. BEYCHOK, in H. NEURATH, *The Proteins*, 2nd Ed., Vol. 2, Academic Press, New York, 1964, p. 261.
18 G. SCATCHARD. J. S. COLEMAN AND A. L. SHEN, *J. Am. Chem. Soc.*, 79 (1957) 12.