Subsite differences between the active centres of papaya peptidase A and papain as revealed by affinity chromatography

Purification of papaya peptidase A by ionic-strength-dependent affinity adsorption on an immobilized peptide inhibitor of papain

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An affinity column consisting of the specific peptide inhibitor of papain, Gly-Gly (*O*-benzyl)Tyr-Arg, attached to Sepharose was found to bind the active thiol proteinase papaya peptidase A specifically, but only at an ionic strength significantly higher than the one at which papain is bound. When a mixture of active papaya peptidase A and its irreversibly oxidized contaminant was applied to the column, the active enzyme was bound whereas the inactive material was not. The bound enzyme was released by deionized water and found to contain 1 mol of SH group/mol of protein. The different conditions required for the binding of the two enzymes to the immobilized peptide was shown to reflect different ionic-strength-dependences of the affinity of the two enzymes for the peptide in solution. Whereas the affinity of papain for the inhibitor appears to be insensitive to ionic strength over the range studied, that of papaya peptidase A is ionic-strength-dependent and always lower than that of papain. A rate assay is devised for papaya peptidase A with *N*-benzyloxycarbonyl-glycine *p*-nitrophenyl ester as the substrate at pH 5.5. After calibration against an active-site titration the assay yields the thiol-group concentration without interference from inactive contaminants. For the papaya peptidase A-catalysed hydrolysis of *N*-benzyloxycarbonyl-glycine *p*-nitrophenyl ester at pH 5.5 k_{cat} was found to be 16.7 s^{-1} , which is about 3 times the value found for the same reaction catalysed by papain.

Preparations of the thiol proteinases papaya peptidase A and papain (EC 3.4.22.2), purified from commercially available dried papaya latex to meet the usual criteria for protein homogeneity, generally contain three enzyme forms: (i) active enzyme in which the essential thiol group is fully reduced, (ii) inactive enzyme in which the thiol group is irreversibly oxidized, and (iii) inactive enzyme that may be activated by reduction, e.g. with dithiothreitol (Glazer & Smith, 1971; Brocklehurst *et al.*, 1981; P. Schack & N. C. Kaarsholm, unpublished work).

Three methods have been devised for the separation of active papain from its inactive components.

Abbreviations used: Gly-Gly-Tyr(OBz)-Arg, glycyl-glycyl-L-(*O*-benzyl)tyrosyl-L-arginine; Z-Gly-ONp, *N*-benzyloxycarbonyl-glycine *p*-nitrophenyl ester.

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In two of the methods purification of the active enzyme is accomplished via covalent attachment of papain through its active-site thiol group to an agarose-bound acceptor, either *p*-aminophenyl-mercuric acetate (Sluyterman & Wijdenes, 1970) or glutathione-2-pyridyl disulphide (Brocklehurst *et al.*, 1973). Protein without a reactive thiol group is not retained by the medium. Treatment with small-*M*_r thiols, which replace the enzyme at the acceptor sites, removes the active enzyme. In the case of the mercurial-agarose species papain can also be removed as the mercury complex by treatment of the medium with HgCl₂. These methods are useful for mixtures of active and inactive forms of a single thiol enzyme. In mixtures of several thiol enzymes, such as for example the papaya latex, none of these two purely chemical methods would obviously be expected to result in the purification of a

single enzyme, although differential purification of thiol enzymes has in fact been accomplished by elution with small- M_r thiols of increasing reducing power (Hillson, 1981).

The third method employs affinity chromatography on a column consisting of a specific peptide inhibitor covalently linked to Sepharose (Blumberg *et al.*, 1970; Funk *et al.*, 1979). The method takes advantage of the preference of papain in catalysing cleavage of peptide sequences at the peptide bond next but one to an aromatic amino acid on its C-terminal side, particularly when the amino acid contributing the carbonyl group to the sensitive bond is arginine, lysine or glutamic acid. Consequently, peptides that have one of these amino acids as their C-terminus and an aromatic amino acid next to it are excellent inhibitors for papain (Schechter & Berger, 1968; Berger & Schechter, 1970). Thus active papain is bound in dilute buffer at low pH to an affinity column of Gly-Gly-Tyr(OBz)-Arg attached to Sepharose, whereas inactive material is not. Purified fully active enzyme is subsequently released by elution with deionized water (Blumberg *et al.*, 1970). Depending on the selectivity of the enzyme specificity, this method may in principle be used to purify a single enzyme from a mixture of otherwise very similar enzymes. Alternatively, the method might be used to evaluate the strength and nature of the inhibitor affinity of individually purified enzymes with suspected similar substrate specificities.

Papaya peptidase A, though definitely a papain-like enzyme, has in recent years been shown to exhibit characteristic differences from papain in significant respects (Brocklehurst *et al.*, 1981; Kaarsholm & Schack, 1983; P. Schack & N. C. Kaarsholm, unpublished work). [While the present paper was in preparation, a renaming of papaya peptidase A as 'papaya peptidase II' was suggested (Brocklehurst & Salih, 1983) in order to link this enzyme with papain (for which the auxiliary name 'papaya peptidase I' was proposed), these being the only two monothiol proteinases in papaya latex.] Thus the two enzymes have been shown to differ with respect to the number of ionizing groups that appear to influence their catalytic action (Baines & Brocklehurst, 1982; Brocklehurst *et al.*, 1983; Schack & Kaarsholm, 1984). Since, unfortunately, no information is available on the structure of papaya peptidase A or its active-centre region, we have used the approach outlined above in a comparative study of the two enzymes in the hope of deducing information on the specificity requirements of papaya peptidase A from those known for papain. The potentiality of the immobilized papain inhibitor Gly-Gly-Tyr(OBz)-Arg as a purification medium for papaya peptidase A has

been explored, and conditions established for the preparation of fully active enzyme. These conditions are found to be explained by significantly different ionic-strength-dependences of the affinity of papain and papaya peptidase A for the inhibitor, which leads to the conclusion that significant differences exist between the active centres of the two enzymes.

Materials and methods

Materials

Dried papaya latex (crude, type 1), papain (2 × crystallized), Z-Gly-ONp, DL-dithiothreitol and 2-mercaptoethanol were obtained from Sigma Chemical Co., St. Louis, MO, U.S.A. CNBr-activated Sepharose 4B and CM-Sepharose CL-6B were the products of Pharmacia, Uppsala, Sweden, and 2,2'-dipyridyl disulphide was from EGA, Steinheim, Germany. Gly-Gly-Tyr(OBz)-Arg was obtained from Vega, Tucson, AZ, U.S.A., and used without further purification. Spray-dried papaya latex was generously given by Powell and Scholefield, Liverpool, U.K. All other chemicals were of analytical grade. Deionized water was used throughout.

Preparation of partly active enzymes

Papaya peptidase A was prepared in a batch procedure from a freeze-dried extract of the commercial dried papaya powder as described previously (Schack, 1967; Kaarsholm & Schack, 1983). Papain was obtained from spray-dried papaya latex by the procedure of Baines & Brocklehurst (1979). Papaya peptidase A was obtained from the same procedure as follows. The supernatant from the papain precipitation [which is 45% saturated with $(\text{NH}_4)_2\text{SO}_4$ (Baines & Brocklehurst, 1979)] was dialysed against 0.05M-NaCl and applied to a 2 cm × 50 cm column of CM-Sepharose CL-6B. From this column papaya peptidase A was eluted as the last enzyme in a linear 0.05–1.0M-NaCl gradient and precipitated by addition of $(\text{NH}_4)_2\text{SO}_4$ to 85% saturation.

Protein concentrations were determined spectrophotometrically by using values of $\epsilon_{280} = 5.6 \times 10^4 \text{ M}^{-1} \cdot \text{cm}^{-1}$ and $\epsilon_{280} = 4.392 \times 10^4 \text{ M}^{-1} \cdot \text{cm}^{-1}$ for papain (Brocklehurst *et al.*, 1973) and papaya peptidase A (Robinson, 1975) respectively. The thiol-group titre of the preparations was determined after activation with dithiothreitol by titration with 2,2'-dipyridyl disulphide at pH 4.0 as described by Brocklehurst & Little (1973). A value of $\epsilon_{343} = 8.08 \times 10^3 \text{ M}^{-1} \cdot \text{cm}^{-1}$ was used for 2-thiopyridone (Stuchbury *et al.*, 1975). The batch-prepared papaya peptidase A and the commercial preparation of papain exhibited a thiol-group content

of typically 0.3–0.4 mol of SH group/mol of protein. The enzyme preparations of spray-dried latex exhibited substantially higher values of thiol-group titre, typically about 0.6–0.8 mol of SH group/mol of protein.

Affinity chromatography

The Sepharose–Gly-Gly-Tyr(OBz)-Arg affinity medium was prepared by the procedure of Blumberg *et al.* (1970). Also, for the purification of papain, the enzyme activation and column elution conditions used by Blumberg *et al.* (1970) were employed.

For the purification of papaya peptidase A typical conditions were as follows. A suspension (1000 μ l) of partly active papaya peptidase A (27 mg of protein/ml) was activated for 45 min with 2000 μ l of activation buffer (20 mM-EDTA/10 mM-2-mercaptoethanol/0.4 M-KCl, pH 4.2). The resulting solution was applied to a 2 cm $\times$ 10 cm affinity column pre-equilibrated with activation buffer. The column was washed with 100 ml of activation buffer followed by 50 ml of 20 mM-EDTA/0.4 M-KCl, pH 4.2. The fully active enzyme was subsequently eluted with deionized water. Fractions of volume 5 ml were collected, and their protein concentration ($A_{280}^{1\%}$) was measured and enzymic activity determined with Z-Gly-ONp as substrate (see below). When not immediately used, the fully active enzyme was treated with a 25% molar excess of HgCl₂ and concentrated on a Diaflo YM-10 membrane (Amicon, Danvers, MA, U.S.A.). When the preparation was stored in this form at 4°C, no loss in potential activity occurred over a period of at least 30 days. Full activity was regained on treatment with a 50-fold molar excess of dithiothreitol at pH 6.

Rate assay for papaya peptidase A

For freshly prepared fully active preparations of papaya peptidase A as well as for partly active preparations of the enzyme, the thiol-group content as determined by titration with 2,2'-dipyridyl disulphide at pH 4.0 was correlated with the enzymic activity in a spectrophotometrical assay similar to that employed by Asboth & Polgar (1977) for papain. To 2800 μ l of 0.1 M-sodium acetate buffer, pH 5.5, containing 1 mM-EDTA, 100 μ l of a 3 mM solution of Z-Gly-ONp in acetonitrile was added. The reaction was started by addition of 100 μ l of enzyme solution, and the absorbance at 340 nm was recorded versus time. The concentration of thiol groups in the reaction cell was about 50 nM. At this concentration, the reaction proceeds at so high a rate that spontaneous hydrolysis can be neglected. The initial velocity was calculated by using a value of $\Delta\epsilon_{340} = 6600 \text{ M}^{-1} \cdot \text{cm}^{-1}$. This value was

found to be essentially constant over the pH range 3.0–5.5.

Inhibition kinetics

Gly-Gly-Tyr(OBz)-Arg was characterized as inhibitor of papaya peptidase A and papain at pH 4.2 at various ionic strengths with Z-Gly-ONp as substrate. In a typical experiment, a sample of the mercury derivative of the enzyme was activated by treatment with a 50-fold molar excess of dithiothreitol in 1 mM-EDTA at pH 6. After 20 min the enzyme was separated from the small- M_r material and transferred into the buffer of the inhibition experiment by ultrafiltration on the YM-10 membrane. For this purpose, five cycles of 10-fold dilution were employed. The thiol group concentration of the preparation was determined by using the rate assay described above. In all inhibition experiments, the thiol-group titre was at least 0.9 mol of SH group/mol of protein. For a fixed enzyme concentration, several concentrations of inhibitor were employed and each of the samples was assayed against several concentrations of substrate in the buffer of the inhibition experiment. Thus results were obtained for different [S], keeping [I] constant, and vice versa. Typical concentrations were [enzyme] = 50 nM, [S] = 20–100 μ M and [I] = 0–700 μ M. From plots of [S]/ v versus [S] and of 1/ v versus [I], the type of inhibition was identified and the inhibition constant estimated.

Results

Affinity chromatography

When a partly active preparation of papain was applied to the affinity column in 20 mM-EDTA/10 mM-2-mercaptoethanol, pH 4.2, the active enzyme was bound whereas the inactive material was not. The bound enzyme was released by deionized water and found to contain 1 mol of SH group/mol of protein. These are the conditions originally prescribed by Blumberg *et al.* (1970) and later used by many others (e.g. Lowe & Whitworth, 1974; Burke *et al.*, 1974). We did not experience problems of the kind reported by Funk *et al.* (1979), who found that both active and inactivatable papain bound to the column under the above conditions, and we have noticed the later appearance of a report on an apparent successful use of the column under those conditions by nearly the same group of investigators (Kimura *et al.*, 1982).

On the other hand, when the mixture of active and inactivatable papaya peptidase A was applied to the column no protein binding occurred under the above conditions, which therefore would not be useful for purification of papaya peptidase A. However, inclusion of 0.4 M-KCl in the application buffer caused active papaya peptidase A to bind to

the column. On the basis of that observation a purification procedure as shown in Fig. 1 was developed. A mixture (with thiol-group titre 0.3 mol of SH group/mol of protein) of papaya peptidase A and its irreversibly oxidized contaminant was activated for 45 min in 20 mM-EDTA/10 mM-2-mercaptoethanol/0.4 M-KCl, pH 4.2, and applied to the affinity column pre-equilibrated in the same buffer. Elution was carried out with this buffer until the non-binding fraction had come off the column. Elution was continued with 20 mM-EDTA/0.4 M-KCl, pH 4.2, in order to remove the mercaptoethanol, after which elution was further carried on with deionized water. The deionized water released fully active papaya peptidase A with a thiol group titre of 1.0 mol of SH group/mol of protein.

In order to rationalize the apparent ionic-strength effect on the binding capacity of the immobilized peptide, studies on the inhibition of papaya peptidase A and papain by the peptide in solution were carried out at pH 4.2 under three conditions: (i) 20 mM-EDTA, (ii) 20 mM-EDTA/0.2 M-KCl and (iii) 20 mM-EDTA/0.4 M-KCl. Results of a typical inhibition experiment are shown in Fig. 2. The inhibition of both enzymes by Gly-Gly-Tyr(OBz)-Arg was found to be competitive under all three conditions, as evidenced by the set of intersecting lines in a $1/v$ -versus- $[I]$ plot, and by the set of parallel lines in an $[S]/v$ -versus- $[S]$ plot. The estimated values of the inhibition dissociation constant, K_i , are given in Table 1. It is seen that the inhibition of papain is the same under all conditions tested and thus is unaffected by variation in ionic strength in that range, and that the tetrapeptide is a substantially weaker inhibitor of papaya pepti-

dase A than of papain at any of the three ionic strengths. Furthermore, the about 4-fold decrease in K_i for papaya peptidase A as a result of the inclusion of 0.4 M-KCl in the 20 mM-EDTA, pH 4.2, solution clearly accounts for the observation that papaya peptidase A could be purified on the affinity column under the conditions of Fig. 1, but not when the protein was applied to the column in 20 mM-EDTA/10 mM-2-mercaptoethanol, pH 4.2.

Rate assay

Fig. 3 shows the relationship between the thiol-group concentration of solutions of papaya peptidase A as determined by titration with 2,2'-dipyridyl disulphide at pH 4.0 and the initial rate of hydrolysis of 100 μ M-Z-Gly-ONp in 1.0 M-acetate buffer, pH 5.5, containing 1 mM-EDTA and 3.3% (v/v) acetonitrile. Identical results were obtained irrespective of whether the enzyme was freshly prepared, fully active or only partly active, indicating that the presence of irreversibly oxidized papaya peptidase A does not affect the assay. Furthermore, a preparation of the mercury derivative of

Table 1. Ionic-strength-dependence of the competitive inhibition constant for the inhibition of papaya peptidase A and papain by Gly-Gly-Tyr(OBz)-Arg at pH 4.2 with Z-Gly-ONp as substrate

For experimental details see the text.

	K_i (μ M)	
	Papaya peptidase A	Papain
20 mM-EDTA	720 $\pm$ 200	32 $\pm$ 10
20 mM-EDTA/0.2 M-KCl	200 $\pm$ 50	39 $\pm$ 12
20 mM-EDTA/0.4 M-KCl	160 $\pm$ 70	39 $\pm$ 5

Fig. 1. Affinity chromatography of papaya peptidase A on a column (2 cm $\times$ 10 cm) of Sepharose coupled with Gly-Gly-Tyr(OBz)-Arg

A suspension (1000 μ l) of papaya A (27 mg/ml) was activated for 45 min with 2000 μ l of activation buffer (20 mM-EDTA/10 mM-2-mercaptoethanol/0.4 M-KCl, pH 4.2). The resulting solution was applied to the column pre-equilibrated with activation buffer. The column was washed with (1) 100 ml of activation buffer followed by (2) 50 ml of 20 mM-EDTA/0.4 M-KCl, pH 4.2. The fully active enzyme was subsequently eluted with (3) deionized water. Flow rate was 200 ml/h. $\bullet$, A_{280} ; $\blacktriangle$, thiol-group titre.

Fig. 2. Inhibition of papaya peptidase A by Gly-Gly-Tyr(Obz)-Arg at various Z-Gly-ONp concentrations in 20 mM-EDTA/0.2 M-KCl, pH 4.2

The different symbols represent different concentrations of inhibitor. The set of parallel lines in (a) combined with the set of intersecting lines in (b) indicate competitive inhibition with $K_i = 200 \mu\text{M}$.

Fig. 3. Relationship between the thiol-group concentration of papaya peptidase A and the initial rate of hydrolysis of $100 \mu\text{M}$ -Z-Gly-ONp in 0.1 M-acetate buffer, pH 5.5, containing 1 M-EDTA and 3.3% (v/v) acetonitrile

For experimental details see the text. Δ , Fully active preparation (1.0 mol of SH group/mol of protein); $\circ$, partly active preparation (0.3 mol of SH group/mol of protein); $\bullet$, mercury derivative of fully active papaya peptidase A in the presence of a 50-fold molar excess of dithiothreitol.

fully active papaya peptidase A that had been stored for 30 days at 4°C was seen to regain full activity when assayed in the presence of a 50-fold molar excess of dithiothreitol. Since the K_m of the reaction is about $9 \mu\text{M}$ at this pH, k_{cat} may be taken as the slope of the line in Fig. 3, yielding a value of 16.7 s^{-1} . When a similar calibration of thiol-group concentration versus initial rate was made for papain, a line of slope 5.4 s^{-1} resulted. As the K_m for the papain-catalysed reaction is $9.3 \mu\text{M}$ at pH 6.8 (Kirsch & Igelström, 1966), the 5.4 s^{-1} may be taken as the k_{cat} value, which means that the specific activity of papaya peptidase A in catalysing the hydrolysis of Z-Gly-ONp at pH 5.5 is about 3 times that of papain.

Discussion

Before consideration of the differences between the supposedly very similar enzymes papain and papaya peptidase A in their affinity for the tetrapeptide Gly-Gly-Tyr(Obz)-Arg, the demonstrated ability of that peptide, when immobilized, to effect a complete separation of the active form of each of these enzymes from their inactive contaminant, considered to differ only by an irreversibly oxidized thiol group, should be noticed. Despite the extensive use of this affinity medium for the purification of papain, the mechanistic basis for this ability appears not to have been much discussed. That the reason for the manifest discrimination by the inhibitor between the active and inactive forms of the enzyme is not obvious and cannot be deduced merely from fulfilment of specificity requirements (cf. Funk *et al.*, 1979) is evidenced by the observation that the binding affinity of even specific inhibitors and substrates for the enzyme appears to be very little affected by the state of the active-site thiol group. In a particular relevant investigation, Lowbridge & Fruton (1974) studied the active site of papain by means of oligopeptide substrates designed according to the known specificity of the enzyme, and from fluorescence measurements they were able to estimate binding constants for the interaction of derivatives of such peptides to active papain as well as to papain inactivated in four different ways by modification of the active-site thiol group. In all cases the binding constants were comparable, and it was concluded that blockage of the active-site thiol group does not prevent the binding of peptides at the extended active site of papain. Even for small specific substrates, such as α -N-benzoyl-L-arginine ethyl ester, Sluyterman (1966) found the dissociation constant for that substrate to the inactive form of papain to be comparable in magnitude with K_m for the hydrolysis of the same substrate by active papain, a finding supported by the observation that K_m esti-

mated for the hydrolysis of the ester catalysed by a low-activity preparation of papain is not significantly altered by the removal of inactive papain (Mole & Horton, 1973). Thus it seems hard to envisage a mechanism for the exclusive binding of the active enzymes in mixture with their inactive forms by the peptide inhibitor, unless a direct participation of the free thiol group in the binding is invoked, besides the specificity of the fit. On that line it is perhaps significant that, although Blumberg *et al.* (1970) asserted that the selectivity of the immobilized peptide towards the active enzyme reflects the finding by Wolthers & Kalk (1970) that only active papain binds inhibitors (*sic*), the latter investigators in fact considered the participation of the active-site thiol group even to the extent of a possible acylation by the inhibitor (Wolthers & Kalk, 1970).

In their study on the proteolytic degradation of the B-chain of oxidized insulin by papain, chymopapain and papaya peptidase A, Johansen & Ottesen (1968) provided clear evidence for differences in the specificity of these three thiol enzymes from the papaya latex. Not only were quantitative differences in the preferences of the enzymes for the various peptide bonds in the substrate demonstrated, but each of the enzymes was found to catalyse the cleavage of one particular bond that was not cleaved by any of the other two. Thus the bond Leu₍₁₇₎-Val₍₁₈₎ was cleaved only by papain, and the bond Leu₍₁₅₎-Tyr₍₁₆₎ only by papaya peptidase A. In the light of the present knowledge of the specificity requirements of papain, based essentially on the work of Berger & Schechter (1970) and referred to above, the position of a tyrosine residue next to the leucine-17 residue contributing the carbonyl group to the sensitive bond is of course the essential feature. Whether the position of alanine-14 in the position in the insulin B-chain sequence, which would correspond to the S₂-subsite of papaya peptidase A, has a similar significance in the specificity of that enzyme is unknown, since so far no study has been reported on the extension or nature of the binding site of papaya peptidase A. The available information seems limited to our finding (Schack & Kaarsholm, 1984) that, in small substrates presenting the benzoyl or the benzyl-oxycarbonyl moiety to the S₂-subsite, papaya peptidase A appears to be insensitive to the nature of the amino acid residue supposedly binding at the S₁-subsite.

In their application of the immobilized Gly-Gly-Tyr(OBz)-Arg peptide to the purification of papain from the bulk of thiol proteinases in papaya latex Burke *et al.* (1974) found that, if the amount of papain sufficed to saturate the column, only that enzyme was bound, and the others passed through. Thus the difference in the affinity of the peptide

for the various papaya enzymes was implicitly acknowledged.

The essential result of the present investigation, that the relative strong affinity of the peptide inhibitor Gly-Gly-Tyr(OBz)-Arg for papain appears to be ionic-strength-independent whereas the weaker affinity for papaya peptidase A exhibits a pronounced ionic-strength-dependence, may be given several interpretations concerning the possible nature of the substrate/inhibitor-binding site of papaya peptidase A. Clearly, in the absence of more stringent information, any such interpretation must be tentative. An obvious one would be that the tetrapeptide is too small to make contact with the specificity determining residues at the active site, i.e. that those residues are more distant to the active-centre cysteine residue in papaya peptidase A than in papain. A more likely interpretation may be that the active-site region of papaya peptidase A is essentially 'featureless' in terms of stereospecific loci demanding complementary fit by the substrate or inhibitor. If the active-site region of papaya peptidase A has a cleft-like structure similar to that known for papain, the difference implied may be just a greater width of that cleft. Alternatively, and indistinguishably, papaya peptidase A may be considered to have its active site on the enzyme surface. On the basis of the conclusion reached by Chothia & Janin (1975) that the driving force for protein-protein associations is hydrophobic interactions, whereas the contribution to be derived from proper formation of hydrogen bonds and Van der Waals' contacts requires complementarity of the surfaces involved, the affinity of papaya peptidase A for substrates and inhibitors may be viewed as being largely due to unspecific hydrophobic interactions, with a much smaller contribution from fulfilment of stereospecific requirements than seen for papain. This would be consistent with the observed effect of increased ionic strength, which is then seen as a salting-out effect of the inhibitor on to the enzyme (and vice versa) (Schrier & Schrier, 1967), adding to the unspecific hydrophobic interaction with the enzyme for which that is essential, but not to the other for which it is less 'in demand'.

Although in the rate assay employed here, with Z-Gly-ONp as the substrate at pH 5.5, we have used the conditions recommended by Asboth & Polgar (1977) for papain, our finding of k_{cat} values of 5.4 s^{-1} and 16.7 s^{-1} for papain and papaya peptidase A respectively is slightly at variance with the report by Polgar (1981) that the specific activities of the two enzymes towards that substrate are similar. More significantly, though, we do not disagree on the implied contention that, despite the apparently greater refinement of the papain molecule in terms of specificity and catalytic mech-

anism, as discussed in this and a previous paper (Schack & Kaarsholm, 1984), the catalytic efficiency of papaya peptidase A is not less than that of papain.

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