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# DEFINITION OF THE SITE OF REACTIVITY OF THE ANCESTRAL PROTEASE OF THE PAPAIN TYPE*

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**Key Word Index**—Protease; sulfhydryl; papain; chymopapain; bromelain; papaya peptidase A; ficin; actinidin; asclepains A and B.

**Abstract**—Digestions of oxidized insulin B chain by the sulfhydryl proteases papain, chymopapain, papaya peptidase A, actinidin, bromelain, ficin and asclepains A and B show a number of similarities in the positions of bond cleavage. It is suggested that these include the site(s) of reactivity of the ancestral proteinase from which these enzymes were descended.

## INTRODUCTION

Sulfhydryl proteases have been isolated from a wide variety of both mono- and dicotyledons. Some of these enzymes and their sources are papain (EC 2.4.22.2), chymopapain (EC 3.4.22.6), papaya peptidase A (all from *papaya*), actinidin (EC 3.4.22.14: from *Actinidia chinensis*), bromelain (EC 3.4.22.4 from *Ananas comosus*), ficin (EC 2.4.22.3: from *Ficus carica*) and asclepains A and B (from *Asceplias syriaca*). Papain and actinidin have recently been shown to have notable homology in their primary structures [1] and to be comparable in tertiary forms [2]. The partially determined sequence of bromelain is also homologous, in large measure, with these two enzymes [3] and other data have shown a high degree of homology of the first thirty residues (which include the essential sulfhydryl group) between papain, chymopapain and papaya peptidase A as well as asclepains A and B ([4, 5] and unpublished work from this laboratory). Ficin has also been shown to be similar to papain at the residues about the essential cysteine and the vital histidine [6]. It is thus reasonable to assume that all of these plant enzymes, which are relatively non-specific in their reactivities, are descended from a common ancestral protease.

The preservation of the ancestral functionality of a protein is probably the reason for the preservation of its structure. Using the series of cysteinyl proteases listed above on a common protein substrate it should be possible to determine at which site(s) the ancestral protease reacted.

For this purpose we have isolated the peptides from digestion of oxidized insulin B chain in the presence of purified papain, chymopapain, papaya peptidase A, bromelain and asclepains A and B [7–11] and compared the points of bond scission with those observed in other laboratories, with the enzymes papain, chymopapain, papaya peptidase A, ficin and actinidin. While differences were observed between the sites of bond scissions, there were notable similarities.

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## RESULTS AND DISCUSSION

The digestion of oxidized insulin B chain was carried out as described previously [11] and the peptide products were separated by either TLC or HPLC and analysed [11].

Data from this laboratory and from others have been combined in Table 1, where cleavage points in insulin B chain are shown in a scaled system where △ represents a single cleavage, ▲ two cleavages and ▲ three or more scissions at the indicated peptide bond. Quantification of the data from the variety of sources employed in compiling Table 1 is not possible. While the reaction conditions used varied somewhat, in general all digestions were done in alkaline pH (except with actinidin [12]), between 30° and 35° for, commonly, extended hydrolysis. The ratio of enzyme to substrate was usually about 1% except for one digestion [13] with chymopapain when it was 4%.

As might be expected from data of the nature of that discussed here, collected from digestions under a variety of conditions, complete accord on the sites of scission for any given enzyme was not found. However, there was sufficient compatibility so that the general features of Table 1 can be discussed with confidence.

There were two positions of cleavage where all the enzymes examined reacted, namely between residues 13 and 14 (–glu–ala–) and between residues 25 and 26 (–phe–try–). However, while the latter bond was broken with a range of frequencies depending on the enzyme, the former was hydrolysed to a significant degree by all the proteases examined.

Three other bonds were broken by most of the family of enzymes discussed, namely between residues 3 and 4 (–asn–gln–), 7 and 8 (–cys–gly–) and 26 and 27 (–tyr–thr–). While the second of these was a common site of cleavage (except with bromelain), it was also a site where cleavage was infrequent. Ficin apparently did not catalyse hydrolysis at either the residue 3/4 bond or that between residues 26 and 27. Similarly asclepain B was without effect on the former of these bonds, and bromelain at the latter. It should also be noted that the three sites under discussion were attacked with a wide range of frequencies. Thus, the bond between residues 26 and 27 was frequently

Table 1. Cleavage points on the insulin B chain hydrolysed by cysteinyl proteases: ▲—major; ▲—lesser; △—minor

| Protease* | Phe | val | asn | gln | 5 his | leu | cys $SO_3H$ | gly | ser | 10 his | leu | val | glu | ala | 15 leu |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Papain (a) | | | ▲ | | | △ | △ | | | | | | ▲ | △ | △ |
| (b) | | | ▲ | | | | ▲ | ▲ | | | | | ▲ | △ | △ |
| Chymopapain (a) | | | ▲ | | | | △ | | | | | | ▲ | | |
| (b) | | | ▲ | | | | △ | | | | | | ▲ | | |
| (c) | | | ▲ | | | | △ | | | △ | | | ▲ | ▲ | |
| Pap. pept. A (a) | | | △ | | | | △ | | | | | | ▲ | | |
| (b) | | | △ | | | △ | △ | | | | | | ▲ | | △ |
| Actinidin (d) | | | ▲ | | △ | | △ | △ | | | | ▲ | ▲ | | |
| Ficin (e) | | | | | | | | ▲ | ▲ | | | | ▲ | | |
| Bromelain (a) | △ | △ | ▲ | ▲ | | | | △ | ▲ | | △ | △ | ▲ | | ▲ |
| Asclepain A (a) | △ | | ▲ | △ | | △ | △ | △ | △ | ▲ | ▲ | | ▲ | △ | ▲ |
| Asclepain B (a) | | | | △ | | △ | △ | | △ | ▲ | △ | | ▲ | | ▲ |

| Protease* | tyr | leu | val | cys $SO_3H$ | 20 gly | glu | arg | gly | phe | 25 phe | tyr | thr | pro | lys | 30 ala |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Papain (a) | △ | ▲ | △ | ▲ | △ | | | △ | △ | ▲ | ▲ | | | | |
| (b) | ▲ | ▲ | △ | ▲ | △ | | | △ | △ | ▲ | ▲ | | | | |
| Chymopapain (a) | | | | ▲ | △ | | | △ | △ | ▲ | △ | | | | |
| (b) | | | | ▲ | | | | △ | | △ | △ | | | | |
| (c) | ▲ | | | ▲ | | ▲ | | | | ▲ | ▲ | | | | |
| Pap. pept. A (a) | | | | ▲ | | | | | | ▲ | ▲ | | | | |
| (b) | | | | ▲ | | | | | | ▲ | ▲ | | | | |
| Actinidin (d) | ▲ | | | | | | | ▲ | | ▲ | ▲ | | | | |
| Ficin (e) | ▲ | | | | | | | | △ | ▲ | | | | | |
| Bromelain (a) | ▲ | ▲ | | △ | | | | | ▲ | △ | | | | | |
| Asclepain A (a) | | ▲ | △ | △ | | | | △ | △ | △ | △ | | | | △ |
| Asclepain B (a) | △ | △ | | △ | | | | | ▲ | △ | △ | | | | △ |

*References (a) This laboratory, (b) ref. [14], (c) ref. [13], (d) ref. [12], (e) ref. [15].

broken in the presence of papain and actinidin, but infrequently in the presence of the asclepains.

Within the limits imposed by the necessarily semi-quantitative nature of the data of Table 1, and accepting, as is argued above, that the enzymes employed in this work have a common ancestry, the five cleavage sites described above probably include those preferred by the ancestral protease. In the insulin B chain (which has no significantly ordered three-dimensional structure) the enzyme might reasonably be considered, from the data presented here, to catalyse hydrolysis preferentially between residues 13 and 14.

## EXPERIMENTAL

All reagents used were of analytical grade unless otherwise noted. Oxidized insulin B chain was obtained from Schwartz-Mann Laboratories, Orangeburg, NY.

The enzymes used were purified by methods previously described: papain, chymopapain and the papaya peptidase A [7, 8]; bromelain (fraction III [9]); asclepains A and B [10].

The digestion of the insulin B chain was performed as has been described [11].

Peptides were separated by two methods: (a) The combination of thin-layer electrophoresis and chromatography described elsewhere in preparing peptide digestion maps was employed [11]. The sheets so obtained were sprayed with a 0.05% soln of ninhydrin in $Me_2CO$ for visualization of the peptides which were then scraped off, extracted with 6 M HCl and hydrolysed in the conventional manner. (b) The digests from the enzymes were separated by HPLC with a Vydac reverse phase C18 column (250 × 4.6 nm; 19 μm particle, 300 Å pore size) equipped with a prefilter. The elution gradient was acetonitrile in 10 mM ammonium acetate, pH 6.5, and was produced with Beckman models 110A and 100A metering pumps, at a flow rate of 1.0 ml/min. The peptide products were hydrolysed conventionally in 6 M HCl under vacuum. Amino acid analyses were performed as reported elsewhere [11]. From the analyses of the products of the digestions and the known sequence of the insulin B chain, points of scission could be determined.

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