IDENTIFICATION OF THE IODINE-SENSITIVE TYROSINES IN PORCINE PEPSIN*

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SUMMARY: Porcine pepsin was iodinated at pH 6.0 and 37° with a 13-fold molar excess of [^{125}I] triiodide. Loss of proteolytic activity levelled off at 75 ± 5%, and 2.8 ± 0.1 gram atoms of iodine were incorporated per mole of enzyme. Pepsin, iodinated in this manner, was digested with chymotrypsin. The bulk of the label was located in two peptides with the sequences: Leu-Gly-Gly-Ile-Asp-Ser-Ser-diiodoTyr-Tyr and Ile-Gly-Asp-Glu-Pro-Leu-Asn-iodoTyr. The latter peptide is the N-terminal sequence of pepsin. It is postulated that one or both of these tyrosines may form part of the secondary binding site of pepsin.

Studies of the iodination of pepsin (1) have shown that tyrosine residues are modified with a concomitant loss of activity. Iodination leads to a decrease of protease, peptidase and esterase activities (2). Acetylation of tyrosine residues, in comparison, causes a marked increase in peptidase activity and a decrease in protease activity (2-4). These observations have given rise to speculation that one or more tyrosine residues form part of the extended binding site of pepsin. In this report, we quantitate the iodination of pepsin and identify the reactive residues.

MATERIALS AND METHODS: Porcine pepsin was purchased from Calbiochem and used without further purification. [^{125}I] sodium iodide was obtained from New England Nuclear Corporation in reductant-free alkali and was added to NaI_3 solution (British Drug Houses Ltd.) to give the desired range of specific activity (0.05 - 1.0 x 10^6 cpm/μequivalent I). Chymotrypsin was purchased from Worthington Biochemical Corporation and penicillocarboxypeptidase-S1 was prepared according to the method of Jones and Hofmann (5). All sequencing reagents were from Pierce Chemical Co. Ltd. Pepsin was assayed

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on BSA[+] using the method described for penicillopepsin (6). Reactions with iodine were carried out in 0.1 M phosphate buffer, pH 6.0, 37°, and were stopped by the addition of an excess of thiosulfate. Pepsin concentration during iodination experiments was 1 mg/ml. Samples were counted on a Nuclear Chicago 4218 gamma counter. Digestion of iodinated pepsin with chymotrypsin (1:20; pepsin concentration, 4 mg/ml) was carried out for 8 hours at 37° in 1% ammonium bicarbonate, pH 7.8. Peptides were fractionated on columns of Sephadex G-25 equilibrated with 0.25% acetic acid, pH 3.0. Peptides were further fractionated by paper electrophoresis using the systems of Bennett (7). Iodopeptides were located by autoradiography using Kodak No-Screen Medical X-ray film NS-54T. Peptides were hydrolyzed in 5.7 N HCL in vacuo at 106° for 20-24 hours. Amino acid analyses were carried out on a Beckman 120 C analyzer according to the method of Moore et al. (8). Digestion with penicillocarboxypeptidase-S1 was carried out in 0.2 M pyridine-formate buffer, pH 4.2, 37°, with an approximate enzyme to substrate ratio of 1:30. Reactions were stopped in a boiling water bath and analyzed on a Beckman 120 C analyzer adapted for physiological fluids with the lithium buffers described by Benson et al. (9). MIT and DIT could be resolved on the short column of this machine. Dansyl-Edman sequence determination of each peptide was carried out as described by Gray (10) but scaled down in the manner of Bruton and Hartley (11).

RESULTS

Labelling of the Enzyme: The results of a typical labelling experiment are shown in Figure 1. The solid lines indicate the loss of activity and the incorporation of iodine when an initial 13-fold excess of iodine was added at 0-time. After 30 minutes, activity levelled off to about 25% initial activity and over 2 gram atoms of iodine had been incorporated per mole. The dashed lines show the effects of adding another 13-fold excess of iodine

+Abbreviations used are as follows: BSA, bovine serum albumin; MIT, monoiodotyrosine; DIT, 3,5-diiodotyrosine.

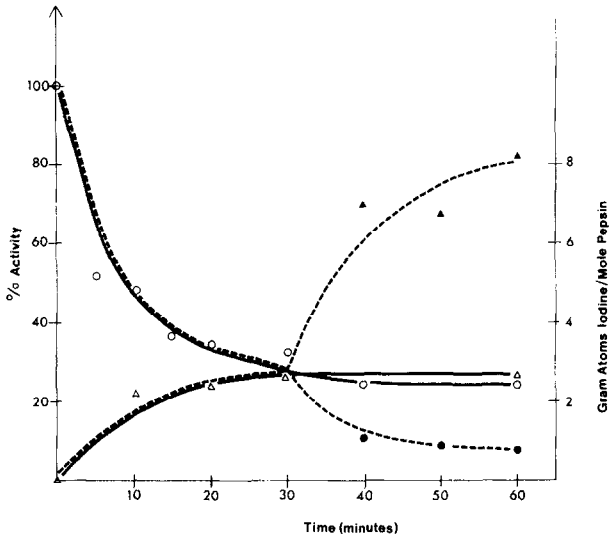

Figure 1. Iodination of pepsin with $^{125}I_3^-$ at 37°, 0.1 M phosphate buffer pH 6.0. Pepsin concentration, 1 mg/ml.
———— 13-fold excess of iodine added at 0 time.
—o—o— loss of activity, —△—△— incorporation of iodine
---------- 13-fold excess of iodine, added at 0 time and at 30 minutes
--●--●-- loss of activity, --▲--▲-- incorporation of iodine

after 30 minutes. Little further inactivation occurs, but there is a much greater incorporation of iodine. From a series of experiments, activity loss was determined as 75 ± 5% and the incorporation of iodine as 2.8 ± 0.1 gram atoms iodine per mole. In a control sample, where thiosulfate had been mixed with the enzyme prior to the addition of iodine, incorporation was 0.004 gram atoms per mole.

Fractionation of Labelled Peptides: A typical fractionation of the peptides obtained from a chymotryptic digest of iodinated pepsin is shown in Figure 2. The distribution of counts eluted from the column is shown in Table I. The material corresponding to peak I was further purified by paper electrophoresis at pH 6.5 and 2.0. From this process an iodopeptide termed 1A3 was isolated. During electrophoresis at pH 6.5, the radioactive band was broadly diffused over the paper, causing substantial losses of material. Peak II was found to be homogeneous as eluted from the column, and was termed peptide II.

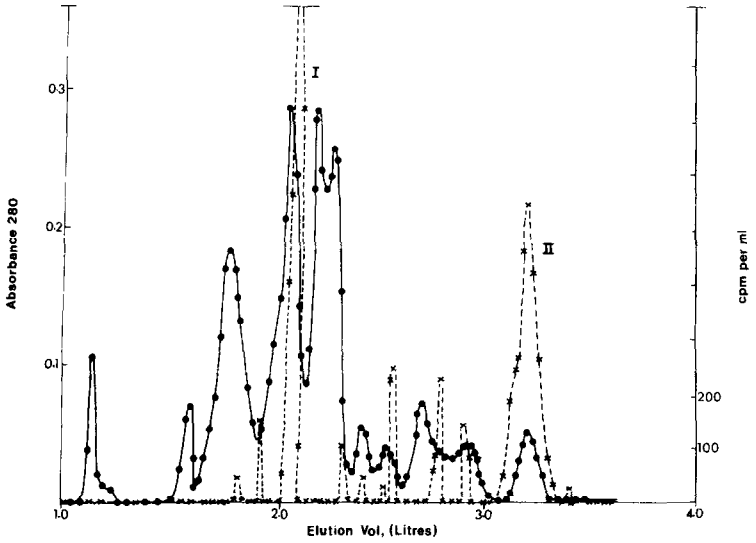

Figure 2. Fractionation of a chymotryptic digest of iodo-pepsin on Sephadex G-25. Column size, 5 x 120 cm; solvent, 0.25% acetic acid, pH 3.0. Fraction size, 5 mls.
--x--x-- cpm per ml; —●—●— Absorbance 280 nm per ml.

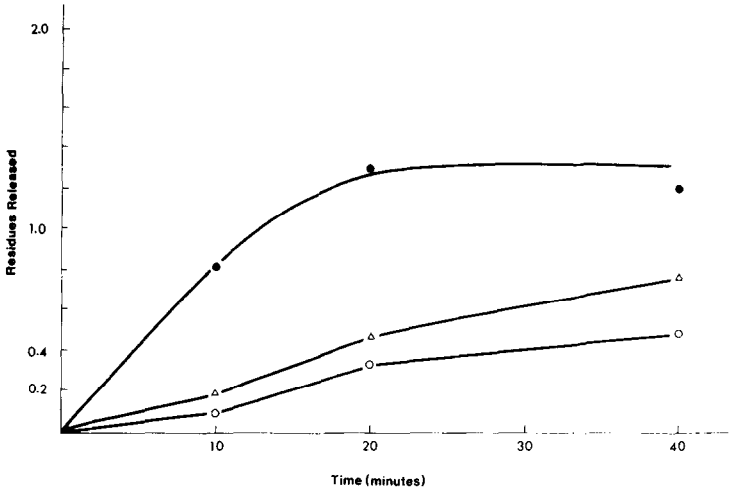

Figure 3. Release of amino acids from peptide II by Penicillocarboxypeptidase-S1.
—●—●— , tyrosine; —△—△— , MIT + DIT; -o-o-, serine.

Iodinated phenols strongly interact with Sephadex gels, particularly at acid pH (12). This, and the hydrophobic character of peptide II, help to explain the fact that this nonapeptide was eluted beyond the gel volume of the column. Other authors (13) have reported the retardation of iodopeptides on Sephadex.

TABLE I. DISTRIBUTION OF COUNTS IN IODINATED PEPSIN.

Fraction	% Total CPM	Gram-atoms I
Peak I	25	0.7
Peak II	55	1.6
Other	20	0.6
Total	100	2.9

TABLE II. AMINO ACID COMPOSITIONS OF IODOPEPTIDES

Amino Acid	nanomoles*	
peptide	IA3	II
Asp	0.010 (2.0)	0.016 (1.1)
Thr	0.001 (0.2)	-
Ser	0.002 (0.4)	0.027 (1.8)
Glu	0.010 (2.0)	0.003 (0.2)
Pro	0.006 (1.2)	-
Gly	0.005 (1.0)	0.030 (1.2)
Val	-	0.005 (0.3)
Ile	0.003 (0.7)	0.014 (0.9)
Leu	0.005 (1.0)	0.015 (1.0)
Tyr	0.002 (0.4)	0.012 (1.4)+
Phe	0.001 (0.2)	-

*values in parentheses are calculated relative to leucine = 1.0.

+some iodotyrosine reverts to tyrosine during acid hydrolysis (1).

Yields of each peptide--uncorrected for losses during purification--were 3% for peptide IA3, and 70% for peptide II. Prior to purification these two peptides accounted for 80% of the iodine incorporated per mole of pepsin.

Identification of the Peptides: The amino acid composition of the peptide IA3 is shown in Table II. Except for some serine, threonine and phenylala-

TABLE III. PENICILLOCARBOXYPEPTIDASE-S1 DIGEST OF PEPTIDE IA3*

Amino acid	Nanomoles released		
	0 min	30 min	60 min
Asn	6.7	6.3	7.9
Glu	5.8	6.6	8.6
Leu	1.6	1.7	2.6
MIT + Tyr	8.6	8.2	8.0

*8.2 nanomoles peptide used per digest.

nine, the composition contained good whole number ratios of amino acids. Dansyl-Edman degradation of peptide IA3 gave the sequence Ile-Gly-Asx-Glx-Pro. Digestion of IA3 with penicillocarboxypeptidase-S1 gave results consistent with a C-terminal sequence of Glu-Asn-MIT (Table III). 70% of the C-terminal amino acid was MIT, 30% was tyrosine. The release of small amounts of threonine, serine and phenylalanine was also observed. Peptide IA3 had a mobility relative to aspartic acid of 0.68 at pH 6.5. This is consistent with a peptide of molecular weight 1136 and a net charge of -3 (14). Thus the sequence of peptide IA3 is:

Ile-Gly-Asp-Glu-Pro-Leu-Glu-Asn-MIT

Peptide II electrophoresed as a single band at both pH 6.5 and pH 2.0. As shown in Table II, except for small amounts of valine and glutamic acid, this peptide appears pure. Dansyl-Edman degradation gave the sequence: Leu-Gly-Gly-Ile-Asx-Ser-Ser-Tyr-Tyr. While the penultimate tyrosine gave only a weak and streaky dansylated derivative, the terminal tyrosine gave a clean and bright spot. This sequence fits the composition of peptide II. Digestion with penicillocarboxypeptidase-S1 gave the results shown in Figure 3 and suggests the terminal sequence Ser-DIT-Tyr. The iodinated tyrosine was roughly 30% MIT and 70% DIT. At pH 6.5, the mobility of peptide II was 0.2 that

of aspartic acid and comparible with a peptide of molecular weight 1100 and a net charge of -1 (14). Thus the sequence of peptide II is:

Leu-Gly-Gly-Ile-Asp-Ser-Ser-DIT-Tyr

DISCUSSION

Both of these peptides have been reported among those fragments of pepsin already sequenced (15). Peptide IA3 is the N-terminal of pepsin. It is highly probable that one or possibly both of these tyrosines are important in the extended binding site of pepsin suggested by other authors (2-4). Studies in this laboratory have shown that many acid proteases, including penicillopepsin, rhizopus-pepsin, and endothia-pepsin, are inactivated by elemental iodine in a manner similar to that of pepsin (Mains and Hofmann, to be published). Since an extended binding site is a common feature of acid proteases (16, 17), it is tempting to speculate that a tyrosine exists in this site in many of these enzymes. Although it is hardly possible at this moment to differentiate the importance of either of these residues with regards substrate binding, the identification of these two sites should provide valuable in the interpretation of the three-dimensional structures of pepsin, penicillopepsin, and other acid proteases.

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