ISOLATION OF THE S-PEPTIDE FORMED ON DIGESTION OF FRUCTOSE 1,6-BISPHOSPHATASE WITH SUBTILISIN AND ITS NON-COVALENT ASSOCIATION WITH THE ENZYME PROTEIN

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SUMMARY: Digestion of rabbit liver fructose 1,6-bisphosphatase with subtilisin results in a several-fold increase in catalytic activity measured at pH 9.2. This change is due to cleavage of a peptide bond located 60 amino acid residues from the NH_2-terminus. The S-peptide and the residual subunit appear as separate peptides in sodium dodecyl sulfate polyacrylamide gel electrophoresis and the S-peptide can be isolated by gel filtration in 9% HCOOH. Under nondissociating conditions, however, the S-peptide remains associated with the protein, and the tetrameric structure and original molecular weight are preserved. Thus the nicking of the peptide chain by subtilisin causes a conformation change that alters the catalytic properties of the enzyme.

We have previously reported that exposure of neutral Fru-P_2ase[1] to subtilisin resulted in the loss of a large peptide from the NH_2-terminus, a shift of the pH optimum from the neutral to the alkaline region, and a loss of sensitivity to the allosteric inhibitor, AMP (1, 2). Similar changes in structure and catalytic properties were observed when the enzyme was incubated with lysosomes (3) or a lysosomal membrane fraction

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1/ Abbreviations: Fru-P_2ase, fructose 1,6-bisphosphatase.

(4, 5). It was assumed that the changes in catalytic properties were the result of the removal of the peptide, since polyacrylamide gel electrophoresis in the presence of Na dodecyl sulfate showed that the molecular weight of the subunits had changed from about 36,000 to 29,000 (1, 2). Indeed kinetic analysis of the changes in subunit structure and the change in activity at pH 9.2 suggested that the digestion with subtilisin was a two-hit process, and that further degradation of the S-peptide was required before it dissociated and produced the changes in catalytic properties (6, 7). We have now isolated and analyzed the S-peptide, which contains 60 amino acid residues with alanine as the COOH-terminus and a blocked NH_2-terminus. We have also found that it does not dissociate under neutral conditions and that the gross molecular weight of the modified protein is not detectably different from that of the untreated enzyme. The change in catalytic properties appears to be due to cleavage of a single peptide bond, without further degradation or dissociation of the S-peptide.

MATERIALS AND METHODS

Fru-P_2ase was purified from livers of New Zealand White rabbits as previously described (8) with minor modifications, including omission of the acid fractionation step. Aldolase purified from rabbit muscle was kindly provided by Dr. C. Y. Lai of this Institute. Hexosephosphate isomerase, glucose 6-phosphate dehydrogenase, triose phosphate isomerase and α-glycerophosphate dehydrogenase were purchased from Boehringer Mannheim Corp., New York, NY. Subtilisin (Carlsberg, Subtilopeptidase A, Type VIII), Fru-P_2-Na_4 salt, NADP, NADH, Na dodecyl sulfate and phenyl methyl sulfonyl fluoride, were obtained from Sigma Chemical Co., St. Louis, MO. [^{14}C] iodoacetic acid was a product of New England Nuclear Corp., Boston, MA. Fluorescamine (Fluram R) was obtained from Hoffmann-La Roche, Inc., Nutley, NJ. Other chemicals were reagent grade. Sephadex G75 was purchased from Pharmacia Fine Chemicals, Inc., Piscataway, NJ.

Fru-P_2ase was assayed at pH 9.2 and 7.5 as previously described with 5 mM $MgCl_2$. Aldolase was assayed as described by Shapiro et al (9). Unless otherwise indicated protein was determined by analysis with fluorescamine (10) after alkaline hydrolysis (11) with the following modification: Aliquots were dried in glass tubes (Corning No. T1285-4, 13 x 100 mm), dissolved in 0.2 ml of 5 N NaOH and autoclaved for 25 min. The samples were cooled, adjusted to pH 8.5 with 0.2 ml of 5 N HCl and 2 ml of 0.5 M borate buffer, pH 8.5, before addition of 0.15 ml of 0.03% fluorescamine in acetone. The method was standardized with bovine serum albumin (Research Products Division, Miles Laboratories, Kankakee, IL). For the chromatography experiments, aliquot of fractions from the column were dried and digested with 0.5 ml of 0.5 N NaOH, and neutralized with 0.5 N HCl. Fluorescence measurements were made with the Aminco-Bowman Spectrophotofluorometer.

Amino acid analyses were carried out by the method of Spackman et al (12) after hydrolysis in 5.7 N HCl in sealed evacuated tubes for 20 h at 110°C. Radioactivity measurements were made with a Beckman Model L-2500 Scintillation Counter, in 10 ml of Aquasol (New England Nuclear Corp., Boston, MA).

Polyacrylamide disc gel and slab gel electrophoresis in Na dodecyl sulfate were carried out as described by Weber and Osborn (13) and Maizel (14), respectively.

RESULTS

Effect of subtilisin on catalytic properties and subunit structure. Digestion of native Fru-P_2ase with subtilisin resulted in a six-fold increase in activity measured at pH 9.2, as previously reported (1, 2), without significant change in the activity measured at pH 7.5 (Fig. 1A). The change in catalytic properties was related to a change in subunit structure; the native subunits, MW = 36,000, were replaced by two peptides having molecular weights of approximately 30,000 and 6,500, respectively, which appeared in polyacrylamide gel electrophoresis in sodium Na dodecyl sulfate (Fig. 1B).

The change in subunit structure, however, did not appear to affect the gross structure of the enzyme. In sucrose density gradient centrifugation, carried out at pH 7.5 or 9.2, (Fig. 2) the molecular weight of the digested enzyme was found to be approximately 144,000, the same as that of the native enzyme, indicating that the peptide remained associated with the modified subunit, and that the tetrameric structure was preserved.

Separation of the S-peptide and modified subunit. When the subtilisin-treated enzyme was filtered through a column of Sephadex G75 under non-denaturing conditions the protein emerged as a single symmetrical peak at or near the excluded volume, with no indication of the presence of fragments (Fig. 3A). It could be shown, however, that this peak contained the S-peptide, which appeared as a separate peak in chromatography in the presence of 9% HCOOH after carboxymethylation of the protein in 8 M urea. In the experiment shown in Fig. 3B, Peak III, containing the S-peptide, accounted for 17.1% of the total protein as determined by analysis with

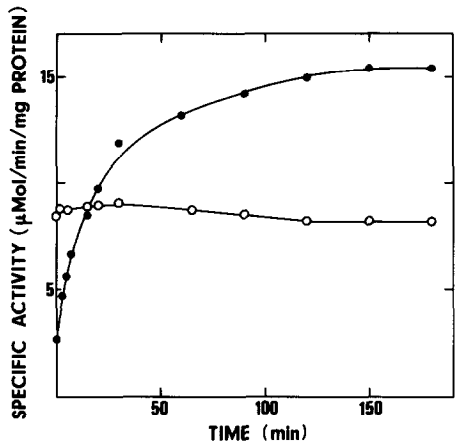

Fig. 1A The effect of digestion with subtilisin on the activity of Fru-P_2ase. The enzyme solution (19.2 mg in 8.2 ml) was dialyzed for 3 h at 2°C against several changes of 0.1 M NH_4Ac, pH 6.5, and incubated with 44.5 μg of subtilisin at 24 ° C. Aliquots were removed as indicated and assayed for activity at pH 9.2 (closed circles) and pH 7.5 (open circles). After 3 h the digestion was stopped by the addition of one-twentieth volume of an ethanolic solution (2 mM) of phenyl methyl sulfonyl fluoride.

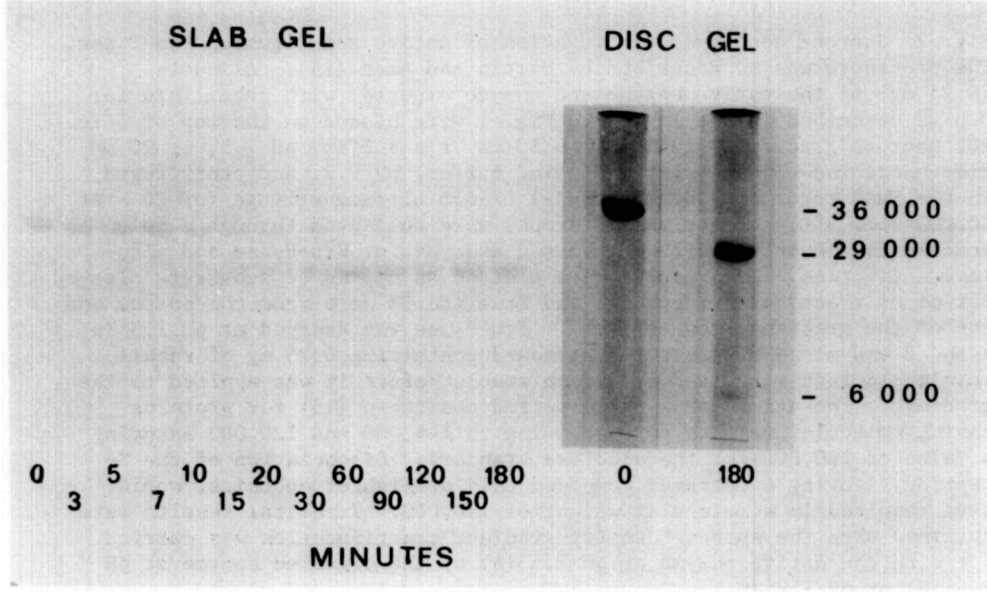

Fig. 1B Changes in subunit structure during digestion with subtilisin. Aliquots (20 μg) were removed before addition of subtilisin at intervals during digestion as indicated. The reaction was stopped by addition of an equal volume of 45% HCOOH, and the samples lyophylized. Flat slab electrophoresis on polyacrylamide gels in Na dodecyl sulfate was carried out with the samples dissolved as described by Maizel (13). The initial sample contained only the 36,000 MW subunit. After 3 h this was almost completely replaced (>95%) by the lighter subunit, MW approximately 30,000. The S-peptide was not visible on the flat plates, but could be seen in disc gel electrophoresis (40 μg aliquot).

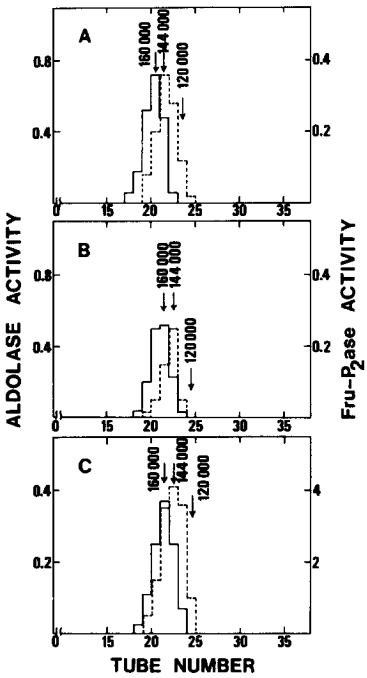

Fig. 2 Sucrose density centrifugation of native and digested Fru-P_2ase. The procedure was as described by Martin and Ames (15). Aliquots (0.25 mg) of the native enzyme and enzyme digested with subtilisin for 3 h as described in the legend to Fig. 1 were placed on the top of 5 to 20% sucrose gradients (13.4 ml) in 10 mM Tris buffer, pH 7.5, or 20 mM diethanolanine--20 mM triethanolamine buffer, pH 9.2, and centrifuged in the SW40 rotor in a Beckman Model L2-65B ultracentrifuge for 20 h at 40,000 rpm, 5°C. Fractions (5 drops) were collected through a hole punched in the bottom of the tube and assayed for Fru-P_2ase and aldolase activities. The results were plotted as μmoles of substrate cleaved per ml of fraction. Fraction 1 and fraction 38 were from the bottom and top of the gradient, respectively. Fru-P_2ase was assayed at pH 7.5 in A and B and at pH 9.2 in C. A standard containing 0.25 mg of rabbit muscle aldolase was mixed with each sample before it was applied to the gradient. The arrows show the expected positions (15) for proteins having molecular weights corresponding to 144,000 and 120,000 assuming a value of 160,000 for the aldolase standard. Dissociation of the S-peptide, leaving a tetramer composed only of lighter subunits, would have resulted in a molecular weight of 118,000. Identical results were obtained when the sucrose density gradient centrifugation was carried out with the native enzyme at pH 7.5 (A) or the digested enzyme at pH 7.5 (B) or pH 9.2 (C).

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fluorescamine after alkaline hydrolysis. Based on the original subunit molecular weight of 36,000, and a mass of 6,300 daltons for the S-peptide (see below), this represents complete recovery of the S-peptide, which must therefore have remained completely associated with the protein

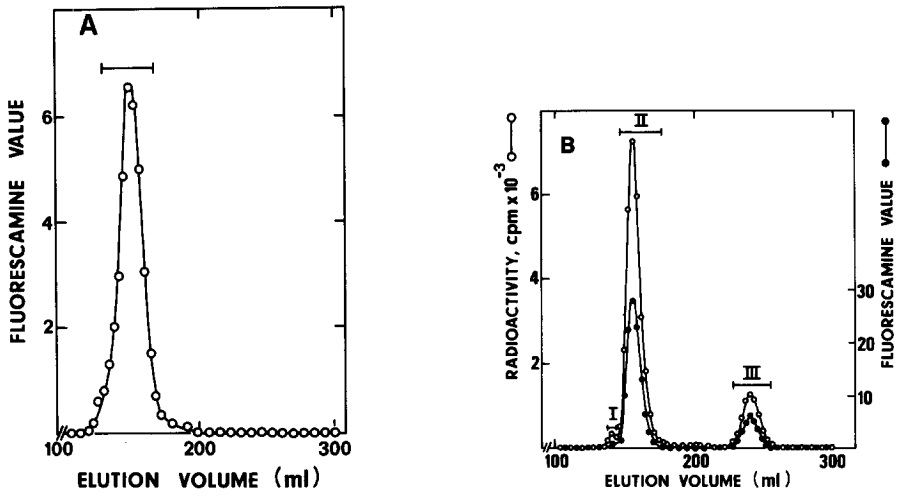

Fig. 3 Chromatography of subtilisin-digested Fru-P_2ase on Sephadex G75 under non-dissociating (A) and dissociating (B) conditions. The sample (8.9 mg), was digested for 3 h as described in the legend to Fig. 1, and after addition of phenylmethyl sulfonyl fluoride, the mixture was placed on a column (1.5 x 195 cm) of Sephadex G75, previously equilibrated at 2°C with 0.1 M NH_4Ac, pH 6.5 (Experiment A). Elution was carried out at 2°C with the same buffer at a flow rate of 14 ml/h. Fractions (3.9 ml) were collected and assayed for protein or peptide with fluorescamine, as described in Materials and Methods. The fractions indicated by the horizontal bar were pooled and lyophylized. Recovery of protein was 8.5 mg.

In Experiment B the pooled lyophylized fractions from Experiment A were dissolved in 2 ml of 8 M urea containing 0.5 M Tris buffer and 26 mM EDTA, pH 8.5, and carboxymethylated with [^{14}C] iodoacetic acid (0.5 mCi/mmol) according to Crestfield et al (16). The carboxymethylated enzyme was dialyzed against 1% HCOOH, brought to 9% HCOOH by addition of 90% HCOOH, and aliquots were taken for determination of protein and radioactivity. The remainder of the solution (2.7 ml, 7.0 mg), was brought to 6 M with solid urea and placed on a Sephadex G75 column (1.5 x 195 cm) previously equilibrated at room temperature with 9% HCOOH. The column was developed with 9% HCOOH, at a flow rate of 15 ml/h. Samples (3.0 ml) were collected and 0.1 ml aliquots assayed for radioactivity. Other aliquots were analyzed for peptide with fluorescamine after alkaline hydrolysis. (See above.) The fractions designated by the horizontal bars were pooled and the radioactivity and peptide content of each peak determined. The total recovery of peptides was 6.3 mg. Peak III accounted for 17.1% of the total peptide, and 16.4% of the total radioactivity.

following cleavage of the peptide bond by subtilisin. This result was confirmed by determination of radioactivity (Fig. 3B). Peak III contained 16.4% of the total radioactivity, as expected for a peptide containing one of the six cysteine residues in the native subunits. (See Table I.)

TABLE I

Amino Acid Composition of S-Peptide

Isolated after Digestion of Fru-P_2ase with Subtilisin

Amino Acid Residue	Moles/Mole Protein*	Amino Acid Residue	Moles/Mole Protein
Lys	3.99 (4)	Gly	5.75 (6)
His	1.00 (1)	Ala	8.69 (9)
Arg	2.95 (3)	Val	2.90 (3)
Cys(Cm)	0.65 (1)	Met	2.85 (3)
Asp	3.85 (4)	Ile	3.85 (4)
Thr	6.55 (7)	Leu	3.97 (4)
Ser	2.85 (3)	Tyr	0.97 (1)
Glu	4.07 (4)	Phe	1.97 (2)
Pro	0.99 (1)	Total	(60)

*Based on the assumption that the peptide contains one histidine residue. The values in parentheses are rounded off to the nearest integral value.

The amino acid composition of the S-peptide is given in Table I. It was found to contain 60 amino acid residues, including one each of histidine, cysteine, proline and tyrosine. The molecular weight calculated from the amino acid composition was 6,300. The COOH-terminal residue was found to be alanine, and the NH_2-terminus was blocked (unpublished observations). Within experimental error, the size of the S-peptide accounts for the change in subunit molecular weight, but the loss of a small peptide from the site of cleavage is not excluded.

DISCUSSION

The results are reminiscent of those obtained by Richards with ribonuclease (17) and Anfinsen and his coworkers with staphylococcal nuclease

(18), in which non-covalent binding of fragments after proteolytic digestion was observed, with preservation of catalytic activity. The experiments described here present two novel and interesting features. One is the fact that Fru-P_2ase is a tetrameric protein, with subunits much larger than the peptide chains in ribonuclease or staphylococcal nuclease. The second is that the catalytic activity, measured at pH 9.2, was substantially increased, and furthermore the sensitivity to AMP, the allosteric inhibitor, was almost completely abolished (1, 2). These changes appear to be related to the presence of a more relaxed or open conformation, because similar changes in catalytic properties were observed when the enzyme was exposed to 5 M urea (2).

In previous experiments with subtilisin the S-peptide appeared to undergo further degradation (6), and the molecular weight of the treated enzyme was estimated to be 120,000 (1), indicating that the S-peptide had indeed been removed. The present results demonstrate that further degradation of the S-peptide is not required for the changes in catalytic properties, which appear to be due to a more relaxed conformation arising from cleavage at a specific locus on the peptide chain.

While this manuscript was in preparation, we learned that J. Zalitis (in press, this journal) had also observed association of S-peptide and protein after digestion of sheep liver Fru-P_2ase with subtilisin. We are indebted to Dr. Zalitis for making these results available to us in advance of publication.

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