MODIFICATION OF NATIVE SHEEP LIVER FRUCTOSE-1,6-BISPHOSPHATASE BY SUBTILISIN*

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SUMMARY

Digestion of native sheep liver fructose-1,6-bisphosphatase by subtilisin resulted in a parallel decrease in activity and sensitivity to AMP inhibition at neutral pH and an increase in specific activity at alkaline pH. During the course of digestion the 35,500 subunit was progressively replaced by two peptides of approximately 29,000 and 6,000 molecular weight, respectively.

A comparison of native and digested fructosebisphosphatase showed no significant changes in molecular weight or tryptophan content; however, their catalytic and regulatory properties were markedly different.

Recently native rabbit liver fructosebisphosphatase (D-fructose-1,6-bisphosphate 1-phosphohydrolase E.C.3.1.3.11), with a neutral pH optimum (1,2), has been modified by digestion with subtilisin (3). The proteolytic conversion of "neutral" to "alkaline" Fru-P_2ase[±] resulted in an increase in specific activity at alkaline pH, a decrease in sensitivity to 5'-AMP inhibition, a loss of tryptophan, and a decrease in molecular weight (3,4). The observed changes appeared to be due to the removal of a large peptide from the native subunit. Native sheep liver Fru-P_2ase (5,6) has a remarkable similarity to the enzyme from rabbit liver in terms

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±Abbreviations: Fru-P_2ase, fructose-1,6-bisphosphatase.
: SDS, sodium dodecylsulfate

of molecular weight, amino acid composition, and tryptophan content. However, upon digestion of native sheep liver Fru-P_2ase with subtilisin, both the time course of the process and the digested enzyme that resulted differed significantly from the results obtained with rabbit liver Fru-P_2ase.

MATERIALS AND METHODS

D-Fructose-1,6 bisphosphate, NADP, 5'-AMP, glucose-6-phosphate dehydrogenase, and phosphoglucoisomerase were purchased from Calbiochem. All buffers and inorganic salts were analytical grade. Subtilisin BPN' was obtained from Teikoku Chemical Industry Co. Ltd., Osaka, Japan. Phosphocellulose (P11) was obtained from Whatman Biochemicals, Maidstone, England.

Native sheep liver Fru-P_2ase was purified from the livers of fed sheep according to the procedure of Chang et al. (6). The purification procedure involved the following: (1) Fresh sheep liver was homogenized in 2 volumes of 0.3 M sucrose, 0.05 M Tris/HCl, 0.1 mM EDTA, pH 7.5, and centrifuged at 12,000 x g for 1 hour. (2) Sufficient phosphocellulose (H^+ form) was added to the liver supernatant to absorb the Fru-P_2ase; the pH was maintained at pH 6.2. The phosphocellulose was eluted batchwise with solutions of 0.1 M Tris acetate and 1mM EDTA, pH 6.0, containing increasing concentrations of KCl. The enzyme was eluted at a concentration of 0.38 - 0.4 M KCl, and the eluates with the highest specific activity were pooled and heat treated at 65°C for 1 min. (3) The enzyme was then concentrated and subjected to column chromatography on phosphocellulose at pH 6.0. Elution was accomplished with a linear gradient from 0 to 0.75 M KCl. Fractions of constant specific activity were pooled and concentrated. The purified enzyme had a specific activity of 42 (units/mg) at pH 7.4 and 20 (units/mg) at pH 9.2 under the assay conditions described. The product was homogeneous by a number of criteria including gel electrophoresis; it contained 4 moles of tryptophan/mole Fru-P_2ase. The detailed purification and characterization of sheep liver Fru-P_2ase will be reported elsewhere. The enzyme was assayed spectrophotometrically by following the rate of NADPH formation at 340 nm in the presence of excess glucose-6P dehydrogenase and glucose-6-P isomerase. One milliliter of the assay mixture at 30° for neutral activity contained 100 mM Tris-HCl, pH 7.4; 1mM $MgSO_4$; 0.5 mM NADP; 0.1 mM fructose-1,6 bisP; 4 units glucose-6P isomerase; 2 units glucose-6-phosphate dehydrogenase; 0.01-0.04 units fructosebisphosphatase (final pH of assay, 7.2). Inhibition at neutral pH was measured in the presence of 10 µM 5'-AMP. Activity at alkaline pH was determined with the assay mixture as described for neutral pH except that 100 mM Tris/HCl, pH 9.5, was used (final pH of assay, 9.2). All enzyme assays contained 0.1 mM EDTA.

Protein was estimated by the method of Lowry et al. (11) with bovine serum albumin as the standard.

Digestion of Fru-P_2ase was carried out as described by Traniello et al. (3) with the following modifications. Fru-P_2ase (9.0mg) was incubated in 2.5 ml 0.01 M potassium acetate, pH 6.5, and digestion was initiated by the addition of 5 µg subtilisin. Samples of 0.1 ml were removed prior to and immediately after subtilisin addition, and then

at the time intervals shown. The samples were added to 0.9 ml ice-cold distilled water; then 0.01 ml of 0.01 M phenylmethylsulfonylchloride was added after 10-15 min a 0.1-ml sample was removed for enzyme assays, and 0.9 ml, (10% w/v) trichloroacetic acid was added to the remaining 0.9 ml.

The precipitates resulting from the trichloroacetic acid treatment were collected by centrifugation, washed two times with 5 ml acetone, dried, and then dissolved in 1 ml of 1% SDS-1% dithiothreitol. Samples containing approximately 25 to 50 μg of protein were analyzed in polyacrylamide gels containing 0.1% SDS prepared and run according to the method of Weber and Osborn (7). Glucagon, cytochrome c, hemoglobin, trypsin, lactate dehydrogenase, ovalbumin, bovine liver glutamate dehydrogenase, bovine serum albumin, and phosphorylase b were treated identically to digestion samples and then used as standards for the estimation of the molecular weight of Fru-P_2ase subunits. Protein bands were stained with Coomassie blue. The gels were scanned in a modified Vitatron densitometer; stain intensity was assumed to be proportional to protein concentration in the gel. Percent of the sample digested was calculated by measuring the areas resulting from the 35,500, 29,000 and 6,000 molecular weight peptide bands in the SDS gels. The values were then substituted in the formula,

$$\% \text{ digestion} = \frac{\text{Area } 29{,}000 + \text{Area } 6{,}000}{\text{Area } 35{,}500 + \text{Area } 29{,}000 + \text{Area } 6{,}000} \times 100$$

Digested Fru-P_2ase was prepared by incubation with subtilisin as described above. After 360 min., 0.1 ml 0.01 M phenylmethylsulfonylchloride was added, and the sample was subjected to phosphocellulose column chromatography with gradient elution. The fractions containing protein were combined, concentrated, and applied to a column of Sephadex G-100.

Tryptophan was estimated on a Beckman 120 C amino acid analyzer (8) after alkaline hydrolysis and by titration with N-bromosuccinimide (12).

Molecular weight determinations were carried out by the meniscus depletion sedimentation equilibrium method as described by Yphantis (10), in a Beckman An-F rotor so that simultaneous determinations could be made on native and digested samples.

RESULTS

The digestion of native sheep liver Fru-P_2ase (1800 parts) by subtilisin (1 part) resulted in the cleavage of one peptide bond. During the time course of digestion, the native subunit of 35,500 was progressively replaced by two peptides of approximately 29,000 and 6,000 molecular weight, respectively (Fig. 1). The ratio of these two peptides remained constant during the course of digestion; this indicates that proteolysis at other sites had not occurred.

The increase in the quantity of 29,000 and 6,000 molecular weight peptides as a result of proteolysis was almost paralleled by an increase in catalytic activity at pH 9.2 (Fig. 2). Concomitant with an

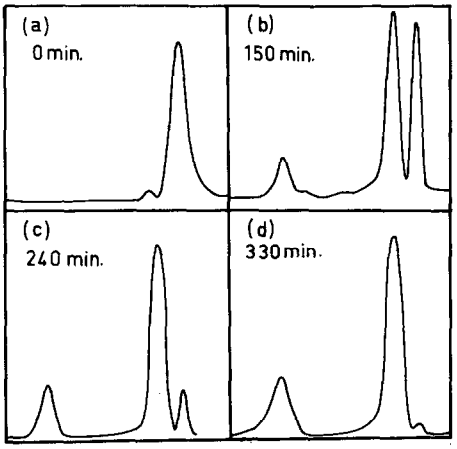

Fig. 1. Tracings of sodium dodecylsulfate gel electrophoresis patterns of sheep liver Fru-P_2ase after (a) 0 min. (b) 150 min. (c) 240 min. and (d) 330 min. digestion with subtilisin. The mobilities of the peaks from right to left corresponded to molecular weights of 35,500; 29,000; and 6,000 respectively.

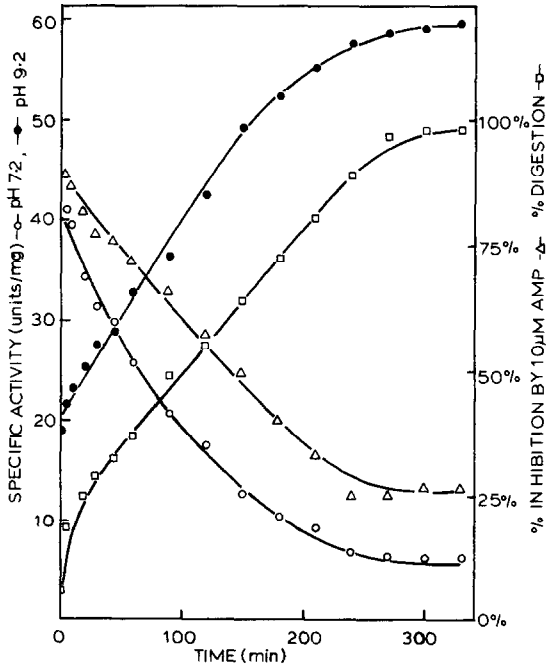

Fig. 2. Digestion of sheep liver Fru-P_2ase with subtilisin. Purified Fru-P_2ase was incubated with subtilisin at 30° in 0.1 M acetate buffer, pH 6.5, at a ratio of enzyme to subtilisin of 1800:1 (w/w). Aliquots were removed at the times indicated and assayed for activity at pH 7.2 in the presence and absence of 10 μM 5'-AMP and for activity at pH 9.2 as described in Methods. Samples were also denatured and analyzed by sodium dodecylsulfate gel electrophoresis.

TABLE 1

Properties of Native and Digested Sheep Liver Fructose-bisphosphatase

PROPERTY	NATIVE	DIGESTED
Specific Activity, pH 7.2 (units/mg)	41.2	5.7
Specific Activity, pH 9.2 (units/mg)	20.1	58.2
Concentration of 5'AMP for 50% inhibition at pH 7.2	4 μm	26 μm
Hill coefficient (n) for 5'-AMP inhibition at pH 7.2*	2.7	1.6
Tryptophan residues/mole Fru-P_2ase**	3.8	4.2
35,500 subunit	96%	3%
29,000 "	4%	74%
6,000 "	-	23%
Molecular weight	142,500±1,400+	144,000++

*Determined according to the method of Taketa and Pogell (14).

**Determined by titration with N-bromosuccinimide (12).

+Mean of 4 determinations.

++Average of 2 determinations.

increased degree of proteolysis there was a parallel decrease in catalytic activity and sensitivity to AMP inhibition at neutral pH.

A comparison of native and subtilisin-degraded fructosebisphosphatase (Table 1) revealed no significant differences in molecular weight and tryptophan content. This indicates that the digested enzyme maintains its quaternary structure and that the 6,000 molecular weight peptide remains bound to the protein. As a result of proteolysis the specific activity decreased at neutral pH and increased at alkaline pH. There was also a marked decrease in sensitivity to AMP inhibition and decreased cooperativity for this effect at neutral pH (Table 1 and Fig. 3).

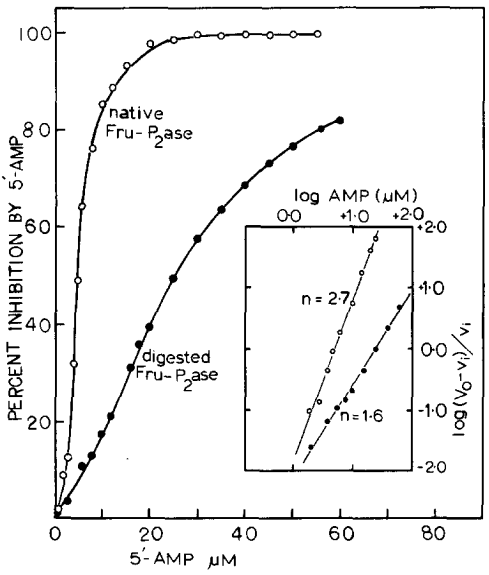

Fig. 3. Inhibition of native (○) and digested (●) fructose-1,6-bisphosphatase by 5'-AMP at pH 7.2. Except for the variable concentrations of 5'-AMP, the assays were performed at pH 7.2 as described in the text. The inset shows the data as Hill plots calculated according to Taketa and Pogell (14); n represents the slope of each line.

DISCUSSION

Despite similarities in molecular weight, subunit composition, amino acid content, and the ratio of neutral to alkaline activity of native rabbit (1,2,3,9) and sheep liver Fru-P_2ase (5,6), the differences in their behavior upon digestion with subtilisin are quite marked. Digestion with subtilisin of neutral rabbit liver Fru-P_2ase prepared from fed animals resulted in increased activity at alkaline pH, decreased sensitivity to AMP inhibition, and a small decrease in activity at neutral pH (3,4,9).

In contrast, controlled digestion of native sheep liver Fru-P_2ase with low concentrations of subtilisin resulted in the cleavage of one peptide bond in the native subunit. The appearance of the 29,000 and 6,000 molecular weight peptides during the time course of digestion

resulted in a parallel increase in activity at alkaline pH and a parallel decrease in activity and sensitivity to AMP inhibition at neutral pH.

The differences between rabbit and sheep liver Fru-P_2ase might be due to the lower enzyme-to-substrate ratio used in our studies, or to inherent differences in sequence, as the sheep liver enzyme appears more sensitive to proteolysis. Thus incubation of sheep liver Fru-P_2ase (200 parts) with subtilisin (1 part) under conditions identical to those described by Traniello et al. (3) for rabbit liver Fru-P_2ase resulted in a rapid increase in alkaline activity and a rapid decrease in neutral activity. After 10 min the alkaline activity had reached a maximum; thereafter both the alkaline and neutral activities decreased. SDS gel electrophoresis revealed that the 29,000 molecular weight peptide was being progressively replaced by peptides of approximately 10,000 and 18,000 molecular weight, respectively (J. Zalitis, unpublished observation). The differences observed may also be due to the use of different subtilisins, viz., subtilisin BPN' in this study and subtilisin Carlsberg in the studies with rabbit liver Fru-P_2ase (3,4,9). Although similar, the two subtilisins show significant differences in specificity (13).

The isolated subtilisin-digested Fru-P_2ase appears to be identical to the native enzyme in both molecular weight and tryptophan content. Therefore the altered catalytic and allosteric properties of subtilisin-digested sheep liver fructose-bisphosphatase appear to be the direct consequence of the cleavage of one peptide bond within the native subunit. The fact that the changes in activity parallel changes in regulatory behavior indicates that the cleavage results in a marked conformational change within the enzyme.

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