Subunit Structure of External Invertase from Saccharomyces cerevisiae

ROBERT B. TRIMBLE AND FRANK MALEY

From the Division of Laboratories and Research, New York State Department of Health, Albany, New York 12201

Because 50% of the mass of the external invertase of Saccharomyces cerevisiae consists of carbohydrate, it has been extremely difficult to obtain an accurate molecular weight of this enzyme by centrifugal or electrophoretic techniques. However, on removing almost all of the oligosaccharide chains of this enzyme with the endo-β-N-acetylglucosaminidase H from Streptomyces plicatus, it has been possible to show that carbohydrate-free invertase is composed of two 60,000-dalton subunits. Terminal sequence analysis with carboxypeptidases A, B, and Y provided strong evidence that the subunits are identical.

Materials and Methods

Invertase Assay

Invertase was assayed at 37°C by measuring the rate of glucose release from sucrose with the Glucostat Special reagent from Worthington Biochemicals Corp. The specific activity is reported as micromol min⁻¹ mg⁻¹.

Invertase Purification

Saccharomyces invertase (β-fructofuranosidase, EC 3.2.1.26) was purified by an abbreviated version of the method of Neumann and Lampen (1). From IH, C strain of Saccharomyces cerevisiae or was purchased from Boehringer Mannheim Corp. as a dry powder containing about 10% enzyme by weight. No differences were observed between the final products from these sources. The following procedure was used to purify the commercial enzyme.

Step 1—Three grams of the dry powder were dissolved in 50 ml of 10 mm sodium phosphate, pH 6.5, and dialyzed exhaustively at 4°C against several changes of buffer. The specific activity of the dialyzed enzyme was 850 units/mg of protein.

Step 2—The supernate from Step 1 (65 ml, 1.4 x 10⁶ units) was passed over a column (1.5 x 18 cm) of Whatman microgranular DE52 cellulose equilibrated at 4°C with 10 mm sodium phosphate, pH 6.5. The column was washed with 150 ml of this buffer and developed with 1.2 liters of a linear NaCl gradient (0 to 0.2 M in 10 mm sodium phosphate, pH 6.5) at a flow rate of 40 ml/h. Invertase activity eluted between 50 and 120 mm NaCl. The column fractions containing invertase with a specific activity greater than 4500 units/mg of protein were pooled and concentrated with an Amicon ultrafiltr.

The concentrated enzyme (5 to 10 mg of protein/ml) was dialyzed exhaustively against distilled water and stored frozen. The DE52 fraction represented 75% of the starting activity (1.1 x 10⁷ units) with a specific activity of 4,700 units/mg of protein.

Step 3—When electrophoresed on SDS-5.25% acrylamide gels, the Step 2 enzyme migrated as a major diffuse protein band ranging in size from 50,000 to 160,000 daltons with a trace contaminant at 40,000 daltons. To remove the latter, 3 ml of the Step 2 enzyme were chromatographed at room temperature on a Bio-Gel P-200 (100 to 200 mesh) column (1.5 x 114 cm) equilibrated with a solution of 10 mm Tris-HCl, 50 mm NaCl, pH 7.1. The enzyme eluted at the column void volume, while the contaminant was retarded. The pooled fractions were lyophilized, dissolved in 3 ml of distilled water, and dialyzed against distilled water. Step 3 invertase migrated as a single, diffuse high molecular weight band on SDS-acrylamide gels and possessed a specific activity of 5100 units/mg of protein.

Endo-β-N-acetylglucosaminidase H

This enzyme was purified to homogeneity from commercial invertase by a procedure similar to that described earlier (2).

Removal of Carbohydrate from Invertase

Carbohydrate was removed from native or (Cm)-invertase by incubating 2 mg of enzyme protein with 0.2 unit of the purified endo-

β-fructofuranosidase in a 1.0 ml reaction volume containing 50 mm sodium citrate, pH 5.5. In the case of (Cm)-invertase, 0.1% SDS was included to solubilize the protein. After 6 or 16 h of incubation at 37°C, the reaction mixture was passed at room temperature over a column (0.9 x 137 cm) of Sepharose 6B equilibrated with 10 mm Tris-HCl, 50 mm NaCl, 0.5% SDS, pH 7.1. This procedure separates the protein moiety from the released carbohydrate chains (3).

Carbohydrate Analysis

The phenolsulfuric acid method (4), with mannose as a standard, was used to detect carbohydrate in column effluents and to determine the presence of total neutral sugars. In some instances, the
enzymatic spectrophotometric assay (5) was used to determine the presence of mannose in acid-hydrolyzed samples.

**Glucosamine Analysis**
Duplicate samples (0.2 to 0.4 mg of protein) were hydrolyzed with 1 ml of 2 N HCl at 110°C for 12 h in evacuated N2-flushed tubes. Free glucosamine was quantitated on an amino acid analyzer using Beckman PA-36 resin with glucosaminitol added as an internal standard to each sample prior to hydrolysis (6). Recovery of the latter was 92 to 98% in all cases.

**Amino Acid Analysis**
Duplicate protein samples (0.1 to 0.3 mg) were hydrolyzed at 110°C in evacuated N2-flushed tubes for 24 h with 1 ml of double-distilled, constant boiling HCl containing 20 µl of redistilled phenol:water (1:10). Analyses were performed on a JEOLCO 5AH automatic amino acid analyzer employing the Durrum DC-1A single column system. Cysteine was quantitated either as (Cm)-cysteine, using invertase carboxymethylated by the method of Kuhn et al. (7), or as cysteic acid (8). For tryptophan analysis, protein samples were hydrolyzed at 115°C for 24 h with 0.3 ml of 6 N methanesulfonic acid (Pierce) in evacuated N2-flushed tubes (9).

The amino acid analysis of invertase, calculated as residues/130,000 g, agrees closely with that of Neumann and Lampen (1). An A410/abs. value of 2.25 mg m⁻¹ l⁻¹ was obtained from the sum of the amino acid residues.

**Ultracentrifuge Analysis**
Sedimentation equilibrium measurements were performed by the meniscus depletion method of Yphantis (10) using a Spinco model E ultracentrifuge equipped with a photoelectric scanner. Analyses were conducted with 100-µl aliquots of carbohydrate-depleted invertase in either 50 mM sodium phosphate, pH 7.1, or 6 M guanidine-HCl at 13,000 and 36,000 rpm, respectively. A partial specific volume, v, of 0.724 ml/g was calculated from the amino acid composition (11). For analysis in guanidine-HCl, v was adjusted to 0.708 as suggested by Lee and Timasheff (12).

**Carboxypeptidase Digestion**
Homogeneous bovine carboxypeptidases A and B and yeast carboxypeptidase Y were kindly provided by Dr. T. H. Flinnair, Jr. of the New York State Department of Health. Procedures for digestion with carboxypeptidases were essentially as described by Ambler (13, 14). (Cm)-invertase (0.7 mg) was dissolved in 0.5 ml of 0.1 M N-ethylmorpholine acetate, pH 8.2, for carboxypeptidases A and B treatment or in 0.1 M pyridine acetate, pH 5.5, for digestion with carboxypeptidase Y. After incubation with a predetermined amount of carboxypeptidase A, B, A + B, or Y for various times at 37°C, the reaction mixtures were passed over a column (1 x 25 cm) of Sephadex G-25 (coarse) equilibrated at room temperature with 20 mM NH4HCO3, pH 7.8. To quantitate recoveries, 13 nmol of norleucine of 85% of its mannose, but, surprisingly, the endoglycosidase-

**RESULTS AND DISCUSSION**

*Enzyme Purification*—The DE52 gradient elution of commercial yeast invertase provides a rapid purification to near homogeneity of large quantities of enzyme. As Fig. 1 shows, the specific activity of invertase is constant through the peak fractions, but its mannose content decreases on the rear side of the elution profile. The heterogeneity of the carbohydrate content of invertase appears to affect its retention by DE52.

![Figure 1](http://www.jbc.org)
amino acid composition but differed in that the slower moving band consistently possessed a greater glucosamine content. Secondly, exhaustive digestion of endoglycosidase-treated invertase with jack bean meal α-mannosidase removed about 80% of its residual mannose, after which the resultant protein migrated as a single, somewhat diffuse band on SDS-acrylamide gels with a molecular weight centered at 63,000. Together these observations suggest that although carbohydrate depletion of invertase greatly improved its resolution on SDS-acrylamide gels, there was still sufficient carbohydrate present to impair the separation of distinct subunits. Efforts to remove additional oligosaccharide chains from carbohydrate-depleted invertase by retreatment with endoglycosidase alone or in the presence of either SDS or mercaptoethanol were unsuccessful, although these procedures were shown previously to aid greatly in the removal of oligosaccharide chains from hen ovalbumin (22) and immunoglobulin M (3).

To enhance the susceptibility of potentially masked oligosaccharides in the native protein to the action of the endoglycosidase, invertase was denatured, and its sulfhydryl groups were carboxymethylated with iodoacetate (7). The resultant protein, although soluble before treatment with the endoglycosidase, came out of solution during the course of digestion with this enzyme. By bringing the reaction mixture to 0.1% with respect to SDS, precipitation of the protein could be prevented without impairing the endoglycosidase.

To compare the kinetics and extent of enzymatic oligosaccharide depletion from both native and (Cm)-invertase, separate endoglycosidase digestion mixtures were prepared, and at various times 10-μl aliquots were withdrawn from each and mixed with 40 μl of electrophoresis sample buffer (16). Prior to electrophoresis, each sample was heated at 100° for 3 min and subjected to discontinuous electrophoresis in a flat plate system containing an SDS-10% acrylamide resolving gel and an SDS-5% acrylamide stacking gel (16). As shown in Fig. 2, native and (Cm)-invertase, before endoglycosidase treatment, display identical diffuse bands ranging from 90,000 to 160,000 daltons. The time course of digestion suggests that oligosaccharide removal from the native enzyme is a slow but progressive process with a family of bands appearing initially between 65,000 and 80,000 daltons and eventually attaining a minimum of 63,000 daltons. In contrast, (Cm)-invertase is rapidly depleted of carbohydrate in only 30 min of endoglycosidase digestion, as indicated by the single sharp band at 63,000 daltons. After 6 h of incubation, the remaining 0.95 ml of each reaction was passed separately through a Sepharose 6B column and analyzed for protein and carbohydrate as depicted in Fig. 3. Comparison of the protein-containing regions clearly demonstrates that carboxymethylation enhances the ability of the endoglycosidase to remove oligosaccharide chains from invertase, as evidenced by the lesser quantity of phenolsulfuric-positive material present in Fig. 3B relative to that in 3A.

Sedimentation Equilibrium Analysis—A portion of the carbohydrate-depleted (Cm)-invertase was dialyzed against 6 M guanidine·HCl and subjected to sedimentation equilibrium analysis. The results revealed a homogeneous species with a molecular weight of 62,800. Because of the limited solubility of (Cm)-invertase under non-denaturing conditions, its molecular weight could not be obtained. However, samples of native invertase, when treated with both endoglycosidase and α-mannosidase, were soluble and yielded a molecular weight of 118,000 ± 1500. The molecular weight of this preparation was reduced to 59,000 in 6 M guanidine·HCl. Thus, the external invertase of yeast appears by this procedure to be composed of two subunits.

Suberimidate Cross-linking—Through the use of dimethyl suberimidate, it is often possible to evaluate on SDS-acrylamide gels the number and type of subunits in an oligomeric protein (18). In the case of carbohydrate-depleted (Cm)-invertase, two bands were obtained, one migrating at 61,800 and the other at 123,000 (not shown). This result is also consistent with invertase being composed of two subunits of identical size.

Residual Carbohydrate Analysis—In order to assess more accurately the sugar composition of invertase fractions, mannose and glucosamine analyses were performed on the native and S-carboxymethylated enzyme before and after endoglycosidase treatment. The results in Table I show 38 glucosamine residues in both the native and (Cm)-invertase which, on the basis of two glucosamines/neural oligosaccharide chain, indicates 18 chains/holoenzyme or 9/subunit. Endoglycosidase treatment of the native enzyme removes seven oligosaccharide chains/subunit, which must be viewed as an average number because of the heterogeneity observed in Fig. 2. All but one oligosaccharide chain is removed by the endoglycosidase from S-carboxymethylated holoenzyme of 120,000 daltons, suggesting that half of the subunits still possess an oligosaccharide chain. By subtracting the weight of the 9 N-acetylglucosamine residues and the small amount of mannose remaining per
Additional details are given in the text. Myces plicatus endoglycosidase as described in Fig. 2. Fractions of 3 ml were collected and their absorbance was measured at 230 nm (A).

Carboxymethylated (B) invertase digested for 6 h with the Streptomyces plicatus endoglycosidase as described under "Materials and Methods." The excellent quantitative recovery of 2 mol of each amino acid released/mol of holoenzyme provides strong evidence for the identity of the invertase subunits.

In conclusion, this report demonstrates, as shown earlier (3, 23-25), that endo-β-N-acetylglucosaminidase H from S. plicatus is a valuable tool for clarifying the structure and composition of glycoproteins. With the aid of this enzyme it has been possible to demonstrate that the invertase from S. cerevisiae is composed of two identical 60,000-dalton subunits, to each of which is added an average of nine neutral oligosaccharide chains consisting (3) of a di-N-acetyllactosamine core and 26 to 54 mannose residues.

Acknowledgments—The expert technical assistance of Ms. Georgina Evans is greatly acknowledged. We thank Dr. R. MacColl for the equilibrium centrifugation analyses and Dr. R. Bellisario and Mr. M. T. Kimmel for providing the amino acid and glucosamine analyses.

**Table I**

| Preparation | Enzyme treatment | Glucosamine | Mannose | Chains/holoenzyme |
|-------------|------------------|-------------|---------|-------------------|
| Native invertase | None | 23.2 | 95 | 4 |
| Endoglycosidase | 21.9 | 89 | 4 |
| α-mannosidase | 18.9 | 66 | 1 |
| (Cm)-Invertase | None | 55.9 | 565 | 18 |
| Endoglycosidase | 18.9 | 66 | 1 |

* Enzyme digestions were for 16 h at 37°C as described under "Materials and Methods."

The subunit, a molecular weight of 60,000 is obtained for carbohydrate-free invertase.

**Subunit Identity and COOH-Terminal Sequence**—To determine whether the subunits are identical in amino acid composition, (Cm)-invertase was subjected to automatic sequence analysis. However, even after 15 coupling cycles, no amino acids were released, suggesting that the NH₂-terminal ends of both subunits are blocked. To circumvent this problem, an analysis of the carboxyl end of invertase was attempted with carboxypeptidases A, B, and Y.

Carboxypeptidase B released 1.94 mol of lysine/mol of invertase and no other detectable residues, indicating that each subunit contained lysine at its COOH terminus. A kinetic study with carboxypeptidase A indicated a terminal sequence of Lys-Val-Glu-(Ser or Gln)-Phe which by the use of a mixture of carboxypeptidases A and B was extended to Arg-Val-Tyr. This sequence was confirmed by a timed digestion with carboxypeptidase Y, which yielded 4 additional residues. The final COOH-terminal sequence and the quantitative recovery of residues, in moles/mole of invertase, obtained with the carboxypeptidases are:

1. Lys-Val-Glu-(Ser or Gln)-Phe-Arg-Val-Tyr,
2. 1.94 2.04 1.91 1.98 2.32 2.01 1.85 2.07

9 to 12

[Leu, Asp, Thr, (Ser or Gln)]

2.6 2.0 1.95 1.83

REFERENCES

1. Neumann, N. P., and Lampen, J. O. (1967) *Biochemistry* 6, 468-475
2. Tarentino, A. L., and Maley, F. (1974) *J. Biol. Chem.* 249, 811-817
3. Tarentino, A. L., Plummer, T. H., Jr., and Maley, F. (1974) *J. Biol. Chem.* 249, 818-824
4. Dubois, M., Gilles, K. A., Hamilton, J. K., Rebers, P. A., and Smith, F. (1956) *Anal. Chem.* 28, 350-356
5. Tarentino, A. L., Plummer, T. H., Jr., and Maley, F. (1970) *J. Biol. Chem.* 245, 4150-4157
6. Plummer, T. H., Jr. (1976) *Anal. Biochem.* 73, 532-534
7. Kuhn, R. W., Walsh, K. A., and Neurath, H. (1974) *Biochemistry* 13, 3871-3877
8. Spencer, R. L., and Wold, F. (1969) *Anal. Biochem.* 32, 185-190
9. Simpson, R. J., Neuberger, M. R., and Liu, T.-Y. (1976) *J. Biol. Chem.* 251, 1936-1940
10. Yphantis, D. (1966) *Biochemistry* 3, 297-317
11. Cohn, E. J., and Edsall, J. T. (1943) in *Proteins, Amino Acids, and Peptides* (Cohn, E. J., and Edsall, J. T., eds) p. 370, Reinhold Publishing Co., New York
12. Lee, J. C., and Timasheff, S. N. (1974) *Arch. Biochem. Biophys.* 165, 268-273
13. Ambler, R. P. (1972) *Methods Enzymol.* 25B, 143-154
14. Ambler, R. P. (1970) *Methods Enzymol.* 25B, 262-272
15. Weber, K., and Osborn, M. (1966) *J. Biol. Chem.* 241, 4406-4412
16. Trimble, R. B., and Maley, F. (1975) *Arch. Biochem. Biophys.* 167, 377-387
17. Laemmli, U. K. (1970) *Nature* 227, 680-685
18. Davies, G. E., and Stark, G. R. (1978) *Proc. Natl. Acad. Sci. U. S. A.* 66, 651-656
19. Carpenter, F. H., and Harrington, K. T. (1972) *J. Biol. Chem.* 247, 5580-5586
20. Gacesa, S., Neumann, N. P., and Lampen, J. O. (1968) *J. Biol. Chem.* 243, 1573-1577
21. Wallace, R. W., Yu, P. H., Dieckert, J. P., and Dieckert, J. W. (1974) *Analog. Biochem.* 61, 86-92
22. Chien, S. F., Yevich, S. J., Li, S.-C., and Li, Y.-T. (1975) *Biochem. Biophys. Res. Commun.* 65, 683-691
23. Tarentino, A. L., Plummer, T. H., Jr., and Maley, F. (1972) *J. Biol. Chem.* 247, 2629-2631
24. Tarentino, A. L., Plummer, T. H., Jr., and Maley, F. (1973) *J. Biol. Chem.* 248, 5547-5548
25. Chen, W. W., Lennarz, W. J., Tarentino, A. L., and Maley, F. (1975) *J. Biol. Chem.* 250, 7006-7013
Subunit structure of external invertase from Saccharomyces cerevisiae.
R B Trimble and F Maley

J. Biol. Chem. 1977, 252:4409-4412.

Access the most updated version of this article at http://www.jbc.org/content/252/12/4409

Alerts:
• When this article is cited
• When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 0 references, 0 of which can be accessed free at http://www.jbc.org/content/252/12/4409.full.html#ref-list-1