and that, on addition of acid or base, exposure takes place of those tryptophyl or tyrosyl residues that are screened in the aggregate. The observation, however, that strongly alkaline conditions are unable to achieve complete exposure of all of the tryptophyl residues, is more in line with the first explanation.

It should be emphasized that these considerations are obviously dependent upon the validity of the assumption that the molar absorptivities derived from difference spectra of model compounds are also applicable for chromophoric residues in the protein perturbed by the various agents employed. If this were not the case, the conclusions would still be qualitatively correct, although the numerical values for the exposed residues might differ somewhat from those presented here. The very rigorous conditions, required to bring all the tryptophyl residues into contact with the surrounding medium, exemplify the high stability of this last level of organized structure in phosphoglucose isomerase. On the basis of presently available data, conclusions regarding the forces responsible for this stability cannot be arrived at. It is hoped that future work on the subunit structure will provide further insight into the complex molecular architecture of this enzyme.

Acknowledgment

We wish to express our gratitude to Dr. J. B. Mudd for generously providing access to his Cary 15 spectrophotometer.

References

Beaven, G. H., and Holiday, E. R. (1952), *Advan. Protein Chem. 7*, 319.
Chatterjee, G. C., and Noltmann, E. A. (1967), *J. Biol. Chem. 242*, 3440.
Donovan, J. W. (1964), *Biochemistry 3*, 67.
Dyson, J. E. D., and Noltmann, E. A. (1969), *Biochemistry 8*, 3533 (this issue; preceding paper).
Frensdorff, H. K., Watson, M. T., and Kauzmann, W. (1953), *J. Am. Chem. Soc. 75*, 5167.
Herskovits, T. T. (1967), *Methods Enzymol. 11*, 748.
Herskovits, T. T., and Laskowski, M., Jr. (1962), *J. Biol. Chem. 237*, 2481.
Herskovits, T. T., and Sorensen, Sr. M. (1968), *Biochemistry 7*, 2523.
Kronman, M. J., Cerankowski, L., and Holmes, L. G. (1965), *Biochemistry 4*, 518.
Kronman, M. J., and Holmes, L. G. (1965), *Biochemistry 4*, 526.
Leonard, W. J., Jr., and Foster, J. F. (1961), *J. Biol. Chem. 236*, PC73.
Noltmann, E. A. (1964), *J. Biol. Chem. 239*, 1545.
Noltmann, E. A. (1966), *Methods Enzymol. 9*, 557.
Polet, H., and Steinhardt, J. (1968), *Biochemistry 7*, 1348.
Simpson, R. B., and Kauzmann, W. (1953), *J. Am. Chem. Soc. 75*, 5139.

The Glycoprotein Structure of Yeast Invertase*

Norbert P. Neumann and J. Oliver Lampen

ABSTRACT: Invertase in yeast appears to exist in two forms. One is intracellular and devoid of carbohydrate. The other is localized externally to the cell membrane in the cell wall and, unlike the internal enzyme, is a glycoprotein which contains approximately 50% carbohydrate. An estimate of both the number and sizes of the carbohydrate units in the enzyme has been obtained by an examination of glycopeptides obtained after proteolytic digestion of the enzyme by *Streptomyce griseus* protease.

The results indicate that approximately 30 chains of polysaccharide of varying size are present per molecule of enzyme. The nature of the carbohydrate–protein linkage has been examined and appears to involve a glucosylaminyl–asparagine bond.

There is evidence that yeast invertase exists in at least two forms which are related to its location in the cell (Gascón and Ottolenghi, 1967; Sutton and Lampen, 1962; Islam and Lampen, 1962; Lampen *et al.*, 1967). The properties of the internal enzyme have been described recently (Gascón and Lampen, 1968; Gascón *et al.*, 1968). The internal enzyme is devoid of detectable carbohydrate. In contrast, the external enzyme which is localized in the cell wall is a glycoprotein containing about 50% carbohydrate (predominantly mannan with a small percentage of glucosamine) (Neumann and Lampen, 1967).

During the past few years the chemical properties of glycoproteins from a variety of sources have been examined. The glycosylamine linkage of the carbohydrate to the amide group of asparagine has been demonstrated or postulated to occur in ovalbumin (Fletcher *et al.*, 1963), γ-globulin (Rosevear and Smith, 1961), α_1-acid glycoprotein (Kamiyama and Schmid, 1962), soybean hemagglutinin (Lis *et al.*, 1966), ovomucoid

* From Rutgers Medical School and the Institute of Microbiology, Rutgers University, The State University of New Jersey, New Brunswick, New Jersey. *Received April 17, 1969.* This investigation was supported by a U. S. Public Health Service Grant A1-04572. Some of the findings were presented at the 50th Annual Meeting of the Federation of American Societies for Experimental Biology, Atlantic City, N. J., April 1966.

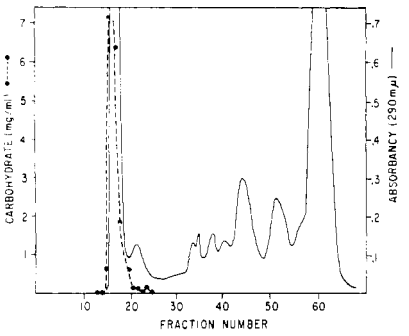

FIGURE 1: Gel filtration of a Pronase digest of 100 mg of invertase on a 2 × 42 cm column of Sephadex G-25. The sample was eluted with 1 M acetic acid in 4-ml fractions.

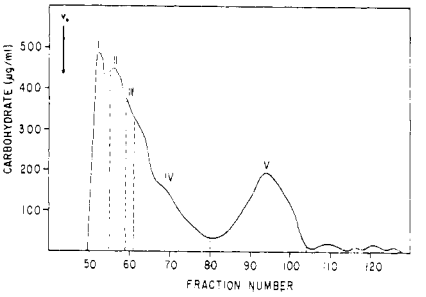

FIGURE 2: Gel filtration of glycopeptide fraction on a 2 × 158 cm column of Sephadex G-50. The sample was eluted with 1 M acetic acid in 5-ml fractions. V_0 is the void volume determined with Dextran Blue.

(Montgomery and Wu, 1963), and ribonuclease B (Plummer *et al.*, 1968). A second type of linkage involving an *O*-glycosidic ether bond to serine and threonine residues has been suggested for mucins (Bhavanandan *et al.*, 1964; Hashimoto and Pigman, 1962) as well as for an enzyme, taka-amylase A (Tsugita and Akabori, 1959).

Recently, Sentandreu and Northcote (1968) isolated a glycopeptide with a molecular weight of 73,000 from yeast cell wall which appears to contain two types of peptide–carbohydrate linkages. One of these connects numerous small monosaccharide or oligosaccharide units to the peptide by glycosyl bonds to the hydroxyl groups of serine and threonine. The other connects a high molecular weight highly branched mannan to the peptide through what is probably a nitrogen glycosyl bond involving *N*-acetylglucosamine and asparagine.

In the present study on yeast invertase, evidence is presented concerning both the number of polysaccharide chains present and the nature of the protein–carbohydrate linkage.

Experimental Procedure

Analyses. Total carbohydrate was measured by the phenol–sulfuric acid method of Dubois *et al.* (1956). Amino acid analyses were performed with a Technicon AutoAnalyzer (Technicon Chromatography Corp., Chauncy, N. Y.). Samples for analysis were hydrolyzed in 6 N HCl at 110° for 20 hr in sealed evacuated tubes (Moore and Stein, 1963). Glucosamine was determined by the ninhydrin method during amino acid analysis. Examination of invertase for the presence of ester bonds was performed by the alkaline hydroxylamine method of Hestrin (1949).

Yeast Invertase. External invertase was isolated from a strain of *Saccharomyces* (mutant FH4C) by a modification of the procedure of Neumann and Lampen (1967). The material used in these studies contained 52% carbohydrate and produced a single band in acrylamide gel electrophoresis at pH 8.6. Preparations of this type have been shown previously to be homogeneous by a number of criteria including electrophoretic, ultracentrifugal, and immunochemical analysis.

Isolation of Glycopeptides. A 100-mg portion of yeast invertase was dissolved in 10 ml of 0.2 M sodium phosphate buffer (pH 7.8). To this was added 0.5 ml of 0.03 M calcium chloride and 1 mg of *S. griseus* protease (Pronase, obtained from Calbiochem, Los Angeles, Calif.) in a volume of 0.1 ml. A drop of toluene was added to inhibit bacterial contamination and the mixture was incubated at 37° for a total of 96 hr. Additional aliquots of Pronase (0.5 mg in 0.05 ml) were added after 48- and 72-hr incubation. The digestion was terminated by the addition of 0.5 ml of glacial acetic acid and the mixture was evaporated to dryness *in vacuo*. The residue was dissolved in 1 M acetic acid to a final volume of 4.7 ml and subjected to gel filtration on a 2 × 42 cm column of Sephadex G-25 (20–80 μ particle size) which had been equilibrated with 1 M acetic acid (Figure 1). The glycopeptide peak (fractions 15–22) was evaporated to dryness and dissolved in 5 ml of water. A small amount of insoluble material was removed by centrifugation, and the supernatant was designated as crude glycopeptide fraction. After removal of aliquots for various analyses, the remainder was passed through a 2 × 158 cm column of Sephadex G-50 (20–80 μ particle size). The material in various tubes was pooled to give five fractions as shown in Figure 2. These fractions were evaporated to dryness, dissolved in 5 ml of water, and their total carbohydrate content and amino acid composition were determined.

Digestion of Invertase with Strong Alkali. Since it seemed possible that certain regions of the invertase molecule might not be susceptible to Pronase action, it was desirable to examine the distribution of polysaccharide chains obtained after complete degradation of the protein moiety. For this purpose 3.2 mg of invertase was dissolved in 0.5 ml of 5 N NaOH in a polypropylene tube and hydrolyzed 16 hr at 110°. The tube was capped with a silicone rubber stopper held in place with a clamp. After hydrolysis, the sample was acidified with 0.15 ml of glacial acetic acid and subjected to gel filtration on Sephadex G-50 (Figure 3).

Hydrazinolysis. Invertase was treated by a procedure similar to that described by Yosizawa *et al.* (1966). In a typical experiment, 10 mg of invertase was dissolved in 0.5 ml of anhydrous hydrazine containing 5 mg of hydrazine sulfate and heated in a sealed tube at 105° for 10 hr. Subsequently, excess hydrazine was removed under reduced pressure by repeated evaporation to dryness with toluene. The hydrazinolysate was dried *in vacuo* over sulfuric acid, dissolved in 1 ml of water, and passed through a column of Sephadex G-25 which had been equilibrated with water. The carbohydrate-containing peak was evaporated to 1 ml and aliquots were analyzed for glucosamine content by the method of Boas (1953) both before and after treatment with sodium borohydride. Release of the glucosamine was effected with 4 N HCl at 100° for 4 hr.

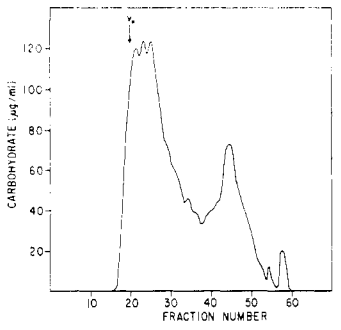

FIGURE 3: Gel filtration of an alkaline digest of invertase on a 0.9 × 45 cm column of Sephadex G-50. The sample was eluted with 1 M NaCl–0.1 M acetic acid in 0.5-ml fractions. V_0 is the void volume determined with Dextran Blue.

Results

Isolation of Glycopeptides from Invertase. The preliminary fractionation of the crude Pronase digest of invertase on Sephadex G-25 is shown in Figure 1. The fastest moving peak contained essentially all of the carbohydrate in the sample and was separated from a large number of other peaks which presumably contain peptides and amino acids. Further fractionation of this partially purified glycopeptide fraction by gel filtration on Sephadex G-50 is shown in Figure 2. A minimum of five carbohydrate-containing peaks was obtained, the first four being incompletely resolved, whereas the fifth was completely resolved from the others. The carbohydrate in these fractions accounted for 68% of the carbohydrate in the starting material. When aliquots of fractions I–IV were pooled and subjected a second time to digestion by Pronase there was only a slight increase in total ninhydrin reactivity and very little change in the gel filtration pattern (no new material eluted in the position of peak V). Thus, cleavage of bonds susceptible to Pronase action was complete after the original multiple treatment with Pronase.

Properties of Glycopeptides. The compositions of the various glycopeptide fractions are shown in Table I. Fraction III is not included in the table because there was insufficient material for adequate analysis. The results are expressed as residues of amino acid relative to aspartic acid (set at one). The predominant amino acids in fraction V are aspartic acid, threonine, and serine, suggesting that these amino acids are localized close to the site of the linkage of carbohydrate to protein in the intact enzyme. The other fractions contain increasing amounts of other amino acids as one progresses from fraction V to I suggesting that these fractions are derived from regions of the invertase molecule more resistant to Pronase digestion.

Examination of Invertase for the Presence of Ester Bonds. Attempts to demonstrate the presence of ester bonds in invertase by the formation of ferric–hydroxamic acid complexes gave results which suggested that hydroxamate formation was occurring due to reaction of hydroxylamine with amide rather than with ester bonds. There was only a slow, small increase in optical density. Thus after 5-min reaction time a value of only 2.1 bonds/molecule was obtained. At 40 min this figure increased to 8.8. Similar results were obtained by Bertolini and Pigman (1967) with bovine serum albumin, orosomucoid, and ovalbumin which do not contain ester bonds.

TABLE I: Composition of Glycopeptide Fractions of Invertase from Pronase Digestion.[a]

Residue	I	II	IV	V
Aspartic acid	1.0	1.0	1.0	1.0
Threonine	0.48	0.80	0.91	0.55
Serine	0.67	0.79	0.73	0.38
Glutamic acid	0.68	0.36	0.36	0.12
Proline	0.34	0.16	0.16	0.13
Glycine	0.48	0.30	0.25	0.23
Alanine	0.44	0.41	0.37	0.16
Valine	0.43	0.16	0.15	0.25
Methionine	0.08	0.06	0.06	0.04
Isoleucine	0.24	0.10	0.08	0.12
Leucine	0.48	0.26	0.21	0.11
Tyrosine	0.39	0.12	0.06	0.06
Phenylalanine	0.50	0.19	0.12	0.07
Lysine	0.40	0.28	0.19	0.09
Histidine	0.18	0.17	0.14	0.08
Arginine	0.19	0.25	0.14	0.05
Glucosamine	0.29	1.11	1.14	1.11
Hexose	7.4	69	32	9.8

[a] Analytical figures given are uncorrected for possible hydrolytic destruction except the value for glucosamine which is corrected for an estimated 57% destruction. This figure is approximate and was obtained previously from analyses of invertase (Neumann and Lampen, 1967). The values are expressed as moles of residue per mole of aspartic acid.

Hydrazinolysis. The over-all recovery of glucosamine prior to reduction by sodium borohydride, was only 35%, indicating extensive destruction by the hydrazinolysis procedure. Numerous attempts to improve these results were unsuccessful. Reduction by sodium borohydride of the carbohydrate units isolated after hydrazinolysis resulted in a further decrease in glucosamine to a level of 17% of the original value. In a control experiment in which invertase was reduced by borohydride without prior hydrazinolysis, there was a complete recovery of glucosamine.

The Effect of Alkali upon Serine and Threonine Residues. In order to test for the presence of *O*-glycosidic bonds, an aliquot of the crude glycopeptide fraction was evaporated to dryness, dissolved in 1 ml of 0.5 N NaOH, and incubated 24 hr at 4° under nitrogen. This was then hydrolyzed in HCl and the amino acid composition was determined and compared with a control sample which had not been subjected to alkali treatment. The recoveries of threonine and serine were 101 and 98%, respectively.

Digestion of Invertase with Strong Alkali. When invertase was digested with 5 N NaOH for 16 hr at 110° the results shown in Figure 3 were obtained. The pattern is complex indicating the release of a number of oligosaccharide chains of varying size. The distribution of material is rather similar to that obtained for the Pronase digest.

Discussion

The results obtained indicate that invertase is a glycoprotein

which contains a large number of polysaccharide chains attached to the protein moiety at various points. Furthermore, these chains vary in size. Gel filtration on Sephadex suggests a molecular weight range of about 7,000–10,000 at one extreme to 2,000–4,000 at the other with varying amounts of material of intermediate sizes in between. At first it was thought that this heterogeneity might be due to incomplete proteolytic digestion by the Pronase. An examination of the gel filtration pattern obtained from the alkaline digest of the enzyme (under conditions where peptide-bond cleavage should have been essentially complete) reveals a pattern somewhat similar to and equally complex as the one obtained after proteolytic digestion.

An estimate of the number of polysaccharide chains is somewhat difficult to obtain in view of the heterogeneity of chain sizes. If one bases it upon the number of residues of aspartic acid recovered in the glycopeptides (corrected for an over-all total yield of 68%), one obtains a value of 47 bonds/molecule assuming that all the aspartic acid is involved in the polysaccharide–protein linkage. In this connection the presence of 38 residues of glucosamine/invertase molecule (Neumann and Lampen, 1967) would set an upper limit for the number of chains if the glucosamine is the linkage point. The real value is lower than this as shown by the results of the hydrazinolysis experiments which indicate that not all of the glucosamine is involved in the linkage. If, on the other hand, one assumes a distribution of the polysaccharide chains into two groups, one with an average molecular weight of 7000 (constituting 75% of the total) and the other with a weight of 3000, a figure of 26 bonds is obtained. Based upon the previous reasoning it is estimated that each invertase molecule contains approximately 30 chains of polysaccharide of varying length.

With regard to the nature of the polysaccharide–protein bond, the two major types which have been demonstrated in other glycoproteins are the glycosylamine linkage to asparagine and the *O*-glycosidic ether bond to serine and threonine. In addition, a third type of bonding, which has been postulated to exist in certain mucoproteins (Graham *et al.*, 1963) but seems unlikely in view of more recent data (Bertolini and Pigman, 1967), is an ester linkage. The inability to demonstrate the presence of significant amounts of ferric hydroxamate chromogen after reaction of invertase with alkaline hydroxlyamine argues against the presence of ester linkages in the molecule.

The results of the hydrazinolysis demonstrate several points. The destruction by sodium borohydride of part of the glucosamine in the carbohydrate units isolated after hydrazinolysis indicates that glucosamine occupies the reducing end of some if not all of the carbohydrate units. Furthermore, the failure to reduce glucosamine by borohydride in the intact enzyme indicates the absence of glucosamine residues with a free aldehydic group. The extensive destruction of glucosamine residues by the hydrazinolysis procedure has limited the usefulness of the technique and our original expectation that this method could be used to quantitate the number of residues of glucosamine involved in protein–carbohydrate linkages was not realized. Following hydrazinolysis of α_1-acid glycoprotein, Yosizawa *et al.* (1966) obtained a much higher over-all recovery of glucosamine and a loss on reduction by borohydride which corresponded to approximately 1 mole/mole of carbohydrate unit. On the basis of this and other data, these authors concluded that the 2-acetamido-1-β-(L-aspartimido)-1,2-dideoxy-D-glucoside linkage is the predominant bond between the carbohydrate and protein moiety of α_1-acid glycoprotein.

The resistance of both the serine and threonine residues of invertase to destruction during treatment with 0.5 N NaOH makes it unlikely that an *O*-glycosidic bond involving these amino acids is present in view of the well-known destruction of these amino acids by the β-elimination reaction which occurs with this type of linkage (Anderson *et al.*, 1964).

The failure to demonstrate the presence of *O*-glycosidic bonding in invertase is interesting in relation to the finding of Sentandreu and Northcote (1968) that in yeast cell wall glycoproteins, *O*-glycosidic bonds carry mannose at a lower degree of polymerization than do bonds involving glucosamine and asparagine. They postulate that the *O*-glucosidic bonds may represent an early stage in the biosynthesis of the large mannan of the cell wall. At a later stage there would be a subsequent transfer of these units by a transglycosylation process to give the high molecular weight material attached to glucosamine and the protein. If this mechanism is correct and if invertase is the end product of a similar type of biosynthetic pathway, it might be expected that invertase would contain few, if any, *O*-glycosidic linkages.

The preponderance of aspartic acid in the isolated glycopeptides suggests that this amino acid is the site of attachment of protein to polysaccharide in invertase. Although the evidence presented is not sufficient to specify the precise nature of the bonding, the results obtained are consistent with the presence of a glycosylamine type of linkage such as has been demonstrated for many other glycoproteins.

Acknowledgment

The authors are indebted to Mrs. L. Schnable and Mr. P. Stein for valuable technical assistance.

Added in Proof

Greiling *et al.* (1969) have recently concluded that the mannan of invertase is linked directly from mannose to the serine or threonine residues of the enzyme protein, based upon extensive destruction of these residues by 0.5 N NaOH.

In view of these results we have reexamined our preparation of invertase for *O*-glycosidic linkages. Attempts to demonstrate β elimination by an increase in absorbancy at 241 mμ in 0.5 N NaOH were unsuccessful. In a separate experiment, invertase was incubated in 0.5 N NaOH at 4° for 162 hr, treated with sodium sulfite, and the sulfonic acid derivatives were measured as cysteic acid (Sentandreu and Northcote, 1968). Under these conditions the appearance of five to six residues per molecule of enzyme was observed, although no decrease in serine or threonine could be detected within the limits of experimental error.

Thus, our preparation of invertase may contain *O*-glycosidic linkages as well as glycosylamine–asparagine bonds although to a much more limited extent than in the preparation of Greiling *et al.* This may reflect the fact that their preparation (from an unidentified strain of yeast) contains a good deal more polysaccharide and has a markedly different amino acid composition than our material.

References

Anderson, B., Seno, N., Sampson, P., Riley, J. G., Hoffman, P., and Meyer, K. (1964), *J. Biol. Chem. 239*, PC 2716.

Bertolini, M., and Pigman, W. (1967), *J. Biol. Chem. 242*, 3776.

Bhavanandan, V. P., Buddecke, E., Carubelli, R., and Gottschalk, A. (1964), *Biochem. Biophys. Res. Commun. 16*, 353.

Boas, N. F. (1953), *J. Biol. Chem. 204*, 553.

Dubois, M., Gilles, K. A., Hamilton, J. K., Rebers, P. A., and Smith, F. (1956), *Anal. Chem. 28*, 350.

Fletcher, A. P., Marks, G. S., Marshall, R. D., and Neuberger, A. (1963), *Biochem. J. 87*, 265.

Gascón, S., and Lampen, J. O. (1968), *J. Biol. Chem. 243*, 1567.

Gascón, S., Neumann, N. P., and Lampen, J. O. (1968), *J. Biol. Chem. 243*, 1573.

Gascón, S., and Ottolenghi, P. (1967), *Compt. Rend. Trav. Lab. Carlsberg 36*, 85.

Graham, E. R. B., Murphy, W. H., and Gottschalk, A. (1963), *Biochim. Biophys. Acta 74*, 222.

Greiling, H., Vögele, P., Kisters, R., and Ohlenbusch, H. D. (1969), *Z. Physiol. Chem. 350*, 517.

Hashimoto, Y., and Pigman, W. (1962), *Ann. N. Y. Acad. Sci. 93*, 541.

Hestrin, S. (1949), *J. Biol. Chem. 180*, 249.

Islam, M. F., and Lampen, J. O. (1962), *Biochim. Biophys. Acta 58*, 294.

Kamiyama, S., and Schmid, K. (1962), *Biochim. Biophys. Acta 63*, 266.

Lampen, J. O., Neumann, N. P., Gascon, S., and Montenecourt, B. S. (1967), *in* Organizational Biosynthesis, Vogel, H. J., Lampen, J. O., and Bryson, V., Ed., New York, N.Y., Academic, p 363.

Lis, H., Sharon, N., and Katchalski, E. (1966), *J. Biol. Chem. 241*, 684.

Montgomery, R., and Wu, Y. C. (1963), *J. Biol. Chem. 238*, 3547.

Moore, S., and Stein, W. H. (1963), *Methods Enzymol. 6*, 819.

Neumann, N. P., and Lampen, J. O. (1967), *Biochemistry 6*, 468.

Plummer, Jr., T. H., Tarentino, A., and Maley, F. (1968), *J. Biol. Chem. 243*, 5158.

Rosevear, J. W., and Smith, E. L. (1961), *J. Biol. Chem. 236*, 425.

Sentandreu, R., and Northcote, D. H. (1968), *Biochem. J. 109*, 419.

Sutton, D. D., and Lampen, J. O. (1962), *Biochim. Biophys. Acta 56*, 303.

Tsugita, A., and Akabori, S. (1959), *J. Biochem. (Tokyo) 46*, 695

Yosizawa, Z., Sato, T., and Schmid, K. (1966), *Biochim. Biophys. Acta 121*, 417.