Glycopeptide Composition of Hepatitis B Surface Antigen

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SUMMARY

Three major polypeptides of hepatitis B surface antigen (HBsAg), with mol. wt. 22,000 (p22), 27,000 (p27) and 68,000 (p68), were separated by preparative SDS-PAGE. These three peptides as well as intact HBsAg were found to have almost identical amino acid compositions and carbohydrate was detected in p27 and p68 by PAS staining. Papain treatment of p68 produced two distinct peptides, p27 and p22. Moreover, when an artificial mixture of p27 and p22 in a ratio of 1:1 was treated with 0.2 M-periodate for 30 min at 37 °C, only p22 was detectable. These results suggest that p68 is composed of p27 and p22, and that p27 is a glycosylated product of p22. Thus, from the evidence obtained, it is possible that p22 (22,000 peptide) is the minimum size of the unique hepatitis B virus (HBV) gene product involved.

INTRODUCTION

The polypeptides of hepatitis B surface antigen (HBsAg) associated with hepatitis B virus (HBV) have been characterized by several workers (Dreesman et al. 1975; Shih & Gerin, 1975, 1977a; Mackay & Burrell, 1976; Peterson et al. 1977; Skelly et al. 1978). However, as far as the number of the peptides is concerned, the results are not consistent; for example the polypeptides were shown to have mol. wt. ranging from 19,000 to 120,000 by SDS-PAGE. Furthermore, accumulated evidence suggests that the HBcAg, HBeAg and DNA polymerase associated with the Dane particle may be virus-coded antigens. Thus, more than ten polypeptides associated with HBV (Robinson, 1977) have been defined at present, which is not consistent with the known genome size (dsDNA, 1.6 × 10^6; Robinson et al. 1974). Therefore, it was of great benefit to determine the mol. wt. of HBsAg, HBcAg, HBeAg and DNA polymerase. In this article we attempt to describe the physico-chemical correlation of the polypeptides of HBsAg to elucidate further the precise structure of this antigen.

METHODS

Purification of HBsAg. Highly purified HBsAg was obtained by the method reported previously (Shiraishi et al. 1978) with minor modifications. In the first step of the purification, affinity chromatography with goat anti-HBs was used (Shiraishi et al. 1974). When 500 ml of antibody-bound Sepharose 4B was added to 5 l of pooled HBsAg-positive donor serum (subtype ad), complete adsorption of HBsAg was found after occasional stirring of the suspension at 4 °C for 48 h. After filtration, the Sepharose was washed with 6 l of 0.01 M-sodium phosphate buffer containing 0.15 M-NaCl, pH 7.2 (PBS), and HBsAg was eluted
with 3 l of 5 M-MgCl₂. The HBsAg-positive fractions (5 l) were dialysed against 20 l PBS for 48 h at 4 °C, and concentrated to 200 ml by an Amicon Hollow Fiber HIX50 apparatus (Amicon Corporation, Lexington, Mass., U.S.A.). The HBsAg fraction was purified further by centrifugation on a pre-formed 10 to 50 % (w/v) KBr gradient at 30000 rev/min for 16 h at 4 °C in a Hitachi RP-42 rotor, followed by rate-zonal sedimentation in glycerol (10 to 50 %, v/v) at 40000 rev/min for 4 h, and KBr (10 to 50 %, w/v) at 30000 rev/min for 16 h. No human serum components were detected by complement fixation tests with rabbit anti-human serum antibodies (Hoechst, Behring Institute, Germany).

Analytical PAGE of purified HBsAg. Samples (30 to 100 µg) were disrupted by heating for 30 min at 60 °C in 0.01 M-sodium phosphate buffer (pH 7.2) containing 8 M-urea, 1 % SDS and 1 % 2-mercaptoethanol (2ME). After addition of 10 % (w/v) sucrose and bromophenol blue (BPB) to the mixture, 50 to 100 µl was layered on to the gel (5 x 80 mm) and electrophoresis carried out for about 5 h at 40 to 50 V (current of 8 mA/gel). The polymerized gels contained 7.5 % acrylamide (made from a stock solution of 22.2 % acrylamide-0.6 % bis), 6 M-urea, 0.07 % (w/v) ammonium persulphate, 0.1 % (v/v) TEMED and 0.1 % (w/v) SDS in 0.1 M-sodium phosphate buffer (pH 7.2). The electrophoresis running buffer consisted of 0.1 M-sodium phosphate buffer containing 0.1 % (w/v) SDS and 0.1 % (v/v) 2ME. After electrophoresis, the proteins were stained with Coomassie brilliant blue (Weber & Osborn, 1969). The carbohydrates associated with the proteins were detected by PAS staining (Glossmann & Neville, 1971). The following proteins were used as standards for the estimation of mol. wt.: phosphorylase b (mol. wt. 94000), bovine serum albumin (mol. wt. 67000), ovalbumin (mol. wt. 43000), carbonic anhydrase (mol. wt. 30000), trypsin inhibitor (mol. wt. 20100) and α-lactalbumin (mol. wt. 14400) (Pharmacia Fine Chemicals, Uppsala, Sweden).

Preparative SDS-PAGE. The separation of the 22000 and 27000 peptides from intact HBsAg was performed by Miles Prep-Disc electrophoresis (Miles Laboratories Inc., Elkhart, Ind., U.S.A.). Samples (7 to 10 mg/2 ml) were solubilized by heating at 60 °C for 30 min in 1 % SDS, 1 % 2ME and 8 M-urea. Sucrose (10 %) and one drop of BPB were added to each solubilized sample and these were then layered on top of polyacrylamide gels and electrophoresis was carried out for about 9 h at 120 to 150 V (constant current of 4 to 8 mA). PAGE was performed in a PD-Z column with a gel vol. of 15 ml and gel length of 10 cm. The polymerized gels contained 7.5 % acrylamide gel (made from a stock solution of 44.4 % acrylamide-1 : 2 % bis), 6 M-urea, 0.07 % (w/v) ammonium persulphate, 0.05 % (v/v) TEMED, 0.1 % (w/v) SDS in 0.4 M-tris-HCl buffer (pH 8.8). The electrophoresis running buffer consisted of 0.02 M-tris-glycine buffer (pH 8.2) containing 0.1 % (w/v) SDS and 0.1 %, 2ME. Elution was carried out with 300 ml of 0.3 M-tris-HCl buffer (pH 8.8) containing 0.1 % SDS and 0.1 %, 2ME, and the elution rate was about 10 ml/h, maintained by using a peristaltic pump P-3 (Pharmacia Fine Chemicals). The eluted proteins were monitored with an ISCO model UA-5.

The separation of the 68000 peptide from the smaller peptides was performed by a plate gel (80 x 80 x 3 mm) electrophoresis apparatus GE-4 (Pharmacia Fine Chemicals), using the same gel conditions as for the analytical SDS-PAGE. After electrophoresis for about 10 h at 50 V, giving a constant current of 100 mA/plate, both the edges and centre of the plate gel were stained with Coomassie brilliant blue to determine the location of the peptides. The gel fractions (50 ml) containing the 68000 peptide were collected and minced with a Sorval omni-mixer after the addition of PBS. Freezing, thawing and sonication were repeated with the minced gel, followed by centrifugation (3000 rev/min for 20 min). The supernatants obtained (300 ml) were concentrated to 20 ml by Amicon 202, PM-10 and then dialysed.
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**Detection methods.** HBsAg was detected by commercial reversed passive haemagglutination (RPHA) test (Antihebcell, Green Cross Co. Ltd., Osaka, Japan). The protein concentration was determined by the method of Lowry *et al.* (1951) using bovine serum albumin as a standard.

**Amino acid analysis.** Lyophilized intact HBsAg and each peptide were hydrolysed in 6 M-HCl at 110 °C for 24 h *in vacuo*. After removal of the acid, the hydrolysates were analysed in a JEOL JLC-8AH amino acid analyser. Half-cystine and cysteine were determined as S-carboxymethyl-cysteine (CM-CySH). The purified HBsAg and each peptide were reduced in 0.01 M-tris-HCl, pH 8.6, containing 0.1 M-dithiothreitol (DTT) and 8 M-urea and alkylated with 0.5 M-iodoacetamide (Crestfield *et al.* 1963).

**Analysis of carbohydrates by gas chromatography-mass fragmentography (GC-MF).** Mannitol was added to each sample as an internal standard and carbohydrates were methylated with m-HCl in anhydrous methanol by heating at 90 °C for 6 h in a sealed tube. After drying out in N₂ gas, samples were further dried over P₂O₅ for 16 h under vacuum. Each sample was redissolved in dry dimethylformamide (DMF) and subjected to trimethylsilylation with pyridine, NO-bis(trimethylsilyl)trifluoroacetamide (BSTFA) and trimethylchlorosilane (TMSC) by heating at 85 °C for 90 min in a sealed tube (Petersson, 1974). With a Shimadzu LKB-9000 gas chromatograph–mass spectrometer, separation was carried out in a 3 m glass column packed with 1% OV-1 (Nihon Chromato Co. Ltd., Tokyo, Japan). The column temperature was 160 °C, and the ionizing potential and filament current were 70 eV and 4 µA, respectively. The carrier gas (He) flow rate was 25 ml/min.

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**RESULTS**

**Polypeptide composition of purified HBsAg**

The purified HBsAg is composed of three main peptides which have mol. wt. of 22 000 (p22), 27 000 (p27) and 68 000 (p68) (Fig. 1). In addition, peptides of mol. wt. 44 000 (p44) and 80 000 (p80) were detected as minor components in some preparations. Two polypeptides (p22 and p27) consistently comprised more than 50% of the total protein content but the relative amounts of p44, p68 and p80 varied among the preparations. Carbohydrate residue was detected in the p27 and p68 peptides by PAS staining (Fig. 1).

**Separation of p22, p27 and p68**

The elution profile of solubilized HBsAg fractionated by preparative SDS-PAGE is shown in Fig. 2. The first peak (fraction 61 to 69) contained p22 and the second peak (fraction 71 to 80), p27. Confirmatory evidence was obtained by SDS-PAGE analysis as shown in Fig. 3 (d, c). As the p68 peptide was found in the late fractions (after fraction 90) (Fig. 2) and had a dispersed distribution, its isolation was attempted separately by plate SDS-PAGE. This resulted in a good recovery as shown in Fig. 3 (b). The amino acid compositions determined for these distinct polypeptides, p22, p27 and p68, and for intact HBsAg were similar (Table 1).

**Proteolytic cleavage of p68**

The relationship between these three polypeptides has been examined by treating p68 with several proteolytic enzymes such as trypsin, papain and pepsin. Only papain gave good results. Purified p68 peptide (0.5 mg/ml) was dialysed against 0.1 M-PBS (pH 7.2) containing 2 mm-EDTA and 0.1% SDS in a vol. of 0.5 ml. Papain (Wako Pure Chemical Industries Ltd., Tokyo, Japan) was dissolved at a concentration of 0.1 mg/ml in 0.1 M-PBS.
Fig. 1. Densitometer scans of SDS–PAGE. (a) Coomassie blue-stained and (b) PAS-stained poly-peptides of intact hepatitis B surface antigen.

Fig. 2. Elution profile of preparative SDS–PAGE using Prep-Disc.

Fig. 3. SDS–PAGE of (a) intact HBsAg and (b) p68, (c) p27 and (d) p22 separated by preparative SDS–PAGE.
Table 1. Amino acid composition of intact HBsAg, p68, p27 and p22

<table>
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Containing 2 mM-EDTA. This was added to 0.5 ml of p68 peptide solution in 10 μl portions and incubated at 37 °C for 5 and 20 min with shaking (Porter, 1959), followed by heating at 100 °C for 5 min. The products were solubilized for 30 min at 60 °C in 1% SDS and 1% 2ME, and tested by SDS-PAGE. Fig. 4 shows that the p68 peptide was degraded into two smaller peptides, p22' and p27', with trace amounts of p44'. It should be noted, however, that the same result was not obtained from the starting preparation of p68, when SDS was first completely removed by dialysis. In summary, three peptides (p22', p27' and p44') were found after papain cleavage of p68.

Periodate treatment of p27 and p22

As it was shown that the p22 and p27 peptides have very similar amino acid compositions, the differences between them were further examined. Equal amounts of p22 and p27, separated by preparative SDS-PAGE, were mixed and used for the experiment. One tenth ml of NaIO4 solution was added to the same amount of peptide solution to make final concentrations of NaIO4 between 0.2 and 0.001 M. After incubation at 37 °C for 30 min with shaking, the reaction was stopped by the addition of 0.05 ml 60% sucrose solution and then dialysed against PBS containing 0.1% sucrose. The reaction mixture was disrupted for 30 min at 60 °C in 1% SDS and 1% 2ME, and subjected to SDS-PAGE. The higher the concentration of periodate used the more definite was the disappearance of fraction p27 (Fig. 5). When 0.2 M-periodate was used, p27 disappeared after 30 min treatment. These results may indicate (i) the susceptibility of p27 to periodate treatment and the resistance of p22, (ii) the loss of p27 fragment on dialysis or (iii) the change of p27 to p22. However, the last hypothesis is unlikely as concurrent increases of the p22 peptide were not found.

Detection of carbohydrate by GC–MF

In a preliminary experiment, when authentic trimethylsilylated (TMSi) derivatives of fucose, mannose and galactose were analysed by GC–MF, the two-carbon fragment, m/e 204.4, was commonly found as was the case with mannitol. This is known to originate for the most part from C-2-C-3 and C-3-C-4. When authentic TMSi ethers of amino sugars, e.g. N-acetylglucosamine were analysed, another two-carbon fragment, m/e 131.2 was detected.
Fig. 4. Densitometer scans of SDS–PAGE, Coomassie blue-stained p68: (a) before treatment with papain, and after treatment with papain at 37 °C for (b) 5 min and (c) 20 min.

Fig. 5. Densitometer scans of SDS–PAGE. Coomassie blue-stained mixtures of p22 and p27 peptides, treated for 30 min at 37 °C by various periodate concentrations: (a) Control, (b) 0·001 M, (c) 0·01 M, (d) 0·05 M, (e) 0·1 M, (f) 0·2 M. The arrows in (b) to (f) show the positions of p22.

(DeJongh et al. 1969). Standard calibration curves for each sugar were obtained by using these fragmented ions. When p27 was analysed according to the above procedure, mannose (8.41 µg/mg protein), galactose (12·06 µg/mg protein), N-acetylg glucosamine (28·35 µg/mg protein) and trace amounts of fucose were found. Although the carbohydrate concentration of p27 was a little lower than that of intact HBsAg, as detected by gas chromatography (Shiraishi et al. 1977), almost the same composition was found.
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**DISCUSSION**

The presence of two major polypeptides of small size in HBsAg, a high degree of similarity between the amino acid composition of these two polypeptides (mol. wt. 27000 and 22000) and the hypothesis that p27 might be a glycosylated product of p22 have been proposed by several workers (Peterson et al. 1977; Shih & Gerin, 1977b; Monjardino & Crawford, 1979). The fact that the highest mol. wt. peptide, 72000, which corresponds to p68 in this article, has a common amino acid composition with the two smaller peptides 29500 and 23000, has also been proposed (Shih & Gerin, 1977b). The possible contamination or the presence of serum proteins such as albumin in HBsAg, however, has not been ruled out (Dreesman & Gerin, 1978). In this work, the amino acid analysis of intact HBsAg as well as p68, p27 and p22 gave very similar results and agreed well with those reported previously (Sukeno et al. 1975; Shih & Gerin, 1977b). This evidence may indicate that the peptide composition of the p68 peptide is closely related to those of the two smaller peptides, p27 and p22, and is probably not contaminated by human serum components. Taking this into account, the restricted digestion of p68 with papain produced three peptides, p44', p27' and p22'. The p22' was constantly produced in higher molar ratio than p27' (data not shown) while p44' could be a dimer of p22'. In addition, the presence of a carbohydrate residue in p68 was consistently found, although the exact composition was not determined in this experiment. The carbohydrate composition of intact HBsAg was first determined by us (Shiraishi et al. 1977) and the result was confirmed by others (Skelly et al. 1978). When the carbohydrate composition of p27 was first examined by GC–MF measurement, mannose, galactose, N-acetylglucosamine and trace amounts of fucose were detected, as found with intact HBsAg.

If we assume p22 is a non-glycosylated component, we may propose that p68 is composed of two molecules of p22 and one molecule of p27. Then the correlation between p22 and p27 should be determined. Although the prevailing hypothesis is that p22 is a non-glycosylated product of p27, the result obtained in this experiment revealed that p27 had a rather higher sensitivity to NaIO₄ than p22. However, an increase of p22 after NaIO₄ treatment of a mixture of p22 and p27 was not definitely obtained. Moreover, the molecular size difference between p22 and p27, i.e. 5000, cannot be explained from the carbohydrate content of p27 (see Results). Thus, another explanation for the mol. wt. difference between p27 and p22 is required, although the amino acid composition of p22 and p27 is almost identical.

In summary, although the relationship between p22 and p27 is not established, it is probable that p68 is composed of p22 and p27, and the hypothesis that HBsAg small spherical particles consist of almost 40 molecules of p68 is proposed (Chaírez et al. 1975). However, the exact determination of the carbohydrate composition of the other two peptides, p22 and p68, is vital in order to establish the hypothesis. Because of a limited supply of p68 and p22, this must await further studies. The simplest explanation for the above results may be that p22 is a minimum translation product of the HBV genome.

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**REFERENCES**


H. SHIRAISHI AND OTHERS


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