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Site-specific labelling of the oligosaccharide chains of antibodies

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Abstract

This paper presents a new method for site-specific labelling of antibodies employing enzymatic reactions without oxidizing or reducing agents. IgG was first treated with immobilized sialidase from *Clostridium perfringens* to cleave bound NeuAc. CMP-9-deoxy-9-salizoyl-NeuAc, an activated sialic acid analogue, was labelled with ^{131}I via the iodogen-method in high yields (>95%). Then the oligosaccharide chains of antibodies were labelled yield with the radioactive NeuAc analogue by transfer using α-2,6-sialyltransferase from rat liver in 50%.

Key words: Sialic acid analogue; Sialyltransferase; Neuraminidase; Iodogen; ^{131}I; Antibody; Site-specific labelling

1. Introduction

During the past decades different methods for the radioactive labelling of antibodies were developed. Labelled antibodies are indispensible as tools in medicine and biochemistry. For each application the immunoreactivity of the antibody plays a decisive role. Therefore, the development of mild reaction conditions for the labelling is of great importance. Besides the random labelling methods, reactions were developed for labelling outside of the antibodies binding site. In those studies two functional groups are involved, the disulphide bridges between the polypeptide chains and the oligosaccharide residues on the Fc-part. In the first procedure, a breaking of disulphide bridges is necessary to obtain thiol groups for the reaction with bifunctional agents [1–3]. The second method is based on oxidation of the saccharide residues to aldehyde groups and subsequent reaction with bifunctional reagents [4–6]. Both conversions influence the immunological properties in a special manner.

In this paper we describe a new way for the labelling by enzymatic transfer of synthetic sialic acid derivatives onto the oligosaccharide chains of antibodies. The advantage of these enzymatic reactions is the absence of oxidizing and reducing agents which guarantees that the antibody structure remains intact. Sialic acids were found in terminal position of oligosaccharide chains of many glycoproteins such as antibodies [7,8]. The enzymes for cleavage (sialidase), activation (CMP-NeuAc synthase) and transfer (sialyltransferase) of sialic acid are available. Several studies with sialyltransferase demonstrated, that differently substituted NeuAc, e.g. 9-amino-9-deoxy-NeuAc and 9-fluoresceinyl-9-deoxy-NeuAc can be transferred onto oligosaccharide chains of glycoproteins [9–16].

2. Materials and methods

2.1. ^{14}C measurement

CMP-*N*-acetyl[4,5,6,7,8,9-^{14}C]neuraminic acid was obtained from Amersham-Buchler. After the reaction, the mixture was separated by gel chromatography and measured with Ready Cups (Beckmann) by a liquid scintillation counter 1215 Rackbeta (LKB Packard). CMP-9-salizoyl-9-deoxy-NeuAc was obtained by reaction of CMP-9-deoxy-9-amino-NeuAc [13] with the *N*-succinimidester of salicylic acid. The detailed synthesis will be described elsewhere.

2.2. ^{131}I iodination

The standard reaction mixture contained 1 mg CMP-9-deoxy-9-salizoyl-NeuAc in 50 mM phosphate buffer pH 7.0. The reaction vials were coated with different quantities of iodogen (Serva). The yield of the labelling reaction was determined by HPLC.

2.3. Chromatography systems

We used a HPLC-system (Merck-Hitachi) with a radioactivity detector LB 507 (Berthold). Chromatography was performed employing a column (15 × 1 cm) of the tentacle ion exchanger EMD TMAE (650) S (MCerck) which was eluted (2 ml/min) with a saline gradient from 0.1 M to 0.7 M containing 15 mM phosphate buffer pH 7.0 within 20 min.

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Abbreviations: NeuAc, *N*-acetyl-D-neuraminic acid; CMP-NeuAc, cytidine-5′-monophospho-NeuAc; α-2,6-ST, β-D-galactosyl-1,4-*N*-acetyl-β-D-glucosamine-α-2,6-*N*-acetylneuraminyltransferase from rat liver (E.C. 2.4.99.1); iodogen, 1,3,4,6-tetrachloro-3α,6α-diphenylglycoluril.

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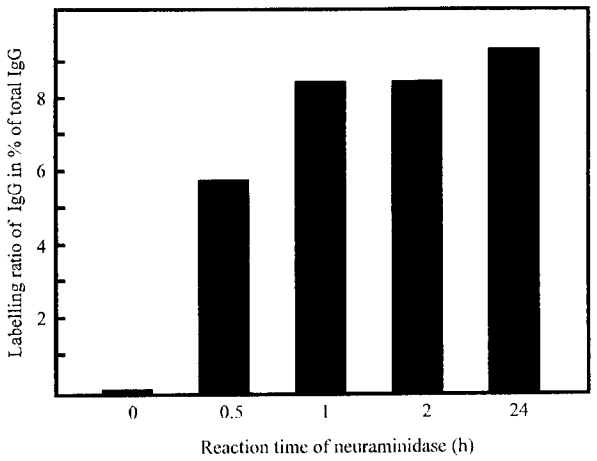

Fig. 1. Transfer of [^{14}C]NeuAc onto IgG after different times of incubation with neuraminidase.

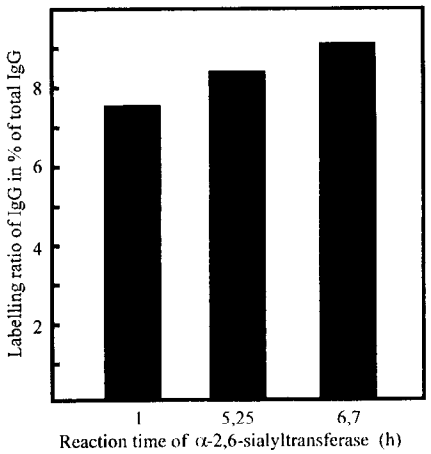

Fig. 2. Introduction of [^{14}C]NeuAc into IgG in dependence on transfer time.

For gel chromatography a standard system (Pharmacia) with a Sephadex G-50 column (20 × 1 cm) was used.

2.4. Enzymatic assays

Desialylation of human IgG was performed at room temperature in 50 mM acetate buffer at pH 5.5 employing immobilized *Clostridium perfringens* sialidase (E.C. 3.2.1.18) (Sigma). The reaction mixture contained 0.2 units of enzyme per milligram IgG and was dialyzed against 50 mM acetate buffer at pH 5.5. Incubation was terminated by centrifugation of the enzyme.

Transfer of the NeuAc-analogues was catalyzed by α-2,6-sialyltransferase (α-2,6-ST) from rat liver (Boehringer Mannheim). For transfer of ^{14}C labeled NeuAc 50 mg IgG, 4.9×10^{-10} mol [^{14}C]CMP-NeuAc and 10 mU α-2,6-ST were incubated.

For the transfer of the monoiodo derivative of CMP-9-deoxy-9-salizoyl-NeuAc 100 mg IgG in 100 ml 15 mM phosphate buffer, pH 7.0, 10 mU α-2,6-ST (5 ml) and 100 ml of the HPLC-fraction containing the radioactive substance were incubated. The reaction mixture for the transfer of the diiodo derivative consisted of 100 mg IgG in 100 ml 15 mM phosphate buffer pH 7.0, 40 mU α-2,6-ST (20 ml) and 200 ml of the HPLC-fraction containing the diiodo derivative.

3. Results and discussion

First we studied the reaction conditions for preparing asialo-IgG and the subsequent transfer of [^{14}C]NeuAc from the CMP-activated compound. Labelling of IgG without pretreatment with neuraminidase was very small (<0.01%). Therefore, IgG was incubated with 0.2 units of immobilized neuraminidase per milligram protein. After different periods of time aliquots of IgG were treated with α-2,6-ST and [^{14}C]CMP-NeuAc. The results show that under these conditions one hour of incubation was sufficient for the removal of protein-bound sialic acid (Fig. 1).

Studying the time-dependence of [^{14}C]NeuAc transfer we found one hour sufficient to label the desialylated IgG (Fig. 2).

Next we labeled CMP-9-deoxy-9-salizoyl-NeuAc with

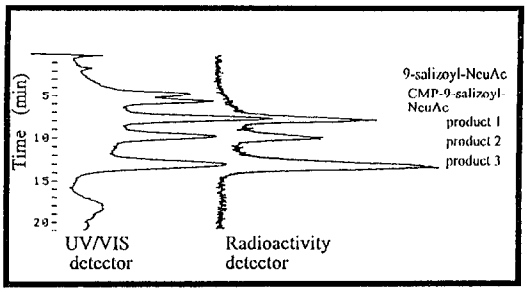

Fig. 3. HPLC-chromatogram of the reaction products 1, 2, and 3 after ^{131}I iodination of CMP-9-salizoyl-NeuAc by iodogen.

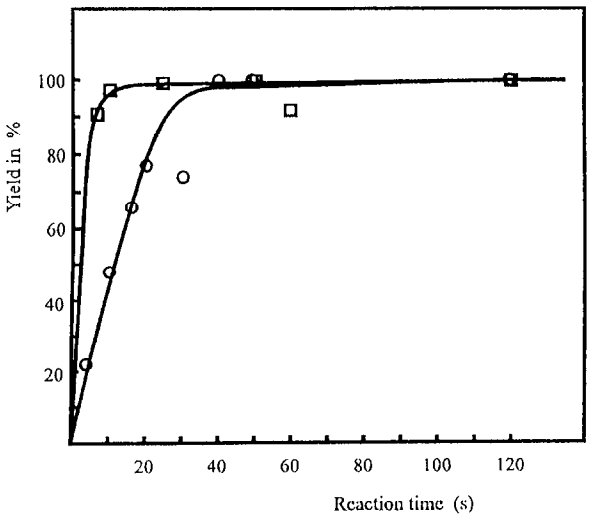

Fig. 4. Time-dependence of the ^{131}I iodination of CMP-9-deoxy-9-salizoyl-NeuAc on two different amounts of iodogen. ○, 1 mg iodogen; □, 10 mg iodogen.

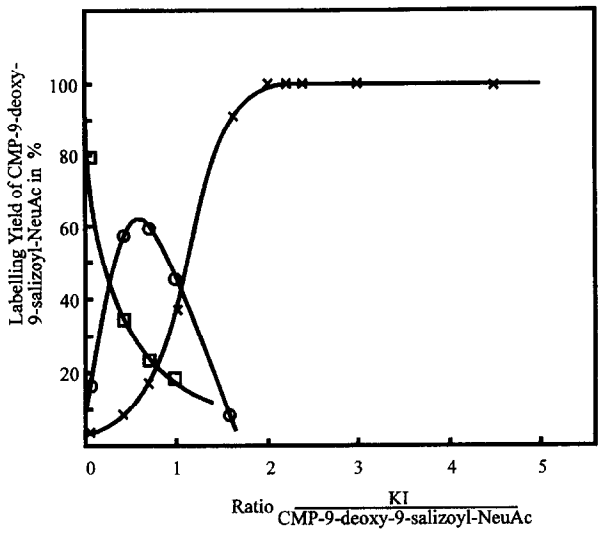

Fig. 5. Influence of different amounts of carrier on the ratio of the reaction products obtained by ^{131}I iodination of CMP-9-salizoyl-NeuAc. □, CMP-9-salizoyl-NeuAc; ○, sum of product 1 and 2; ×, product 3.

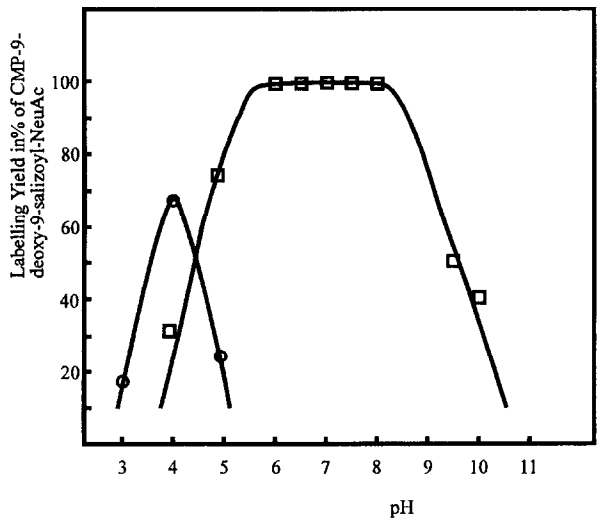

Fig. 6. Dependence of the ^{131}I iodination of CMP-9-salizoyl-NeuAc (carrier added reaction) on the pH-value. ○, monoiodinated products; □, diiodinated product.

^{131}I via the iodogen-method [7]. This procedure was preferred because it is aC solid phase reaction. After separation from the iodogen-coated vial the reaction mixture contained only the starting materials and iodinated products without oxidizing or reducing agents.

Applying HPLC we found three different radioactive products (Fig. 3). Products 1 and 2 were obtained by reaction with carrier-free ^{131}I solution, product 3 by the addition of a carrier KI. The latter one has a higher specific radioactivity than the others. Therefore, apparently 1 and 2 are two different monoiodinated derivatives whereas 3 is diiodinated.

Iodination of the activated sialic acid analogue showed, that the reaction with 1 or 10 mg iodogen was very fast and complete within a few seconds (Fig. 4).

For carrier added iodination we used vials with 100 mg iodogen. Under these conditions the reaction was complete at the same short time. Addition of different amounts of carrier iodide produced different ratios of the three reaction products. As expected, by addition of excess KI only diiodinated sialic acid analogue, product 3, was formed (Fig. 5). This result confirms the formation of two different monoiodinated and one diiodinated products. In all these labelling reactions no decomposition of the CMP-9-deoxy-9-salizoyl-NeuAc by iodogen was observed.

Further, the dependence of the iodogen reaction on the pH was studied. The reaction was complete in the pH-range 6 to 8 (Fig. 6). At lower pH we found only monoiodinated products and free iodine whereas a higher pH caused decomposition giving undefined radioactive and nonradioactive products.

Transfer of ^{131}I labelled CMP-9-deoxy-9-salizoyl-NeuAc: two ^{131}I labelled products were transfered onto human IgG. Fig. 7 shows the transfer of product 1, Fig. 8 the transfer of product 3. The reason for the difference in reaction velocity and yield may be a different kinetics of the α-2,6-ST for the two substrates and the different ratio between α-2,6-ST, IgG and the iodinated product.

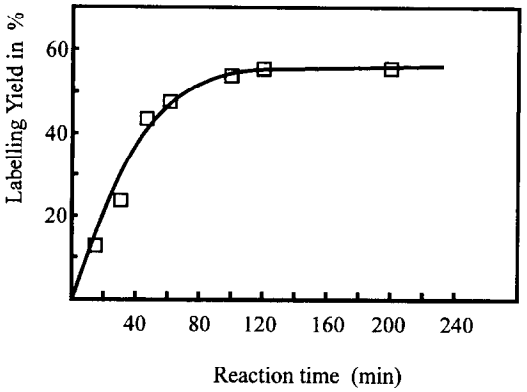

Fig. 7. Transfer of product 1 onto asialo-IgG by α-2,6-sialyltransferase.

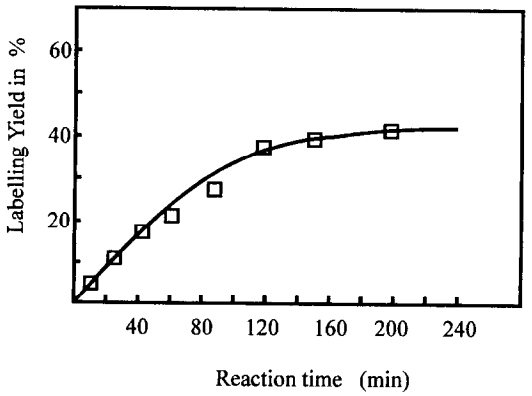

Fig. 8. Transfer of product 3 onto asialo-IgG by α-2,6-sialyltransferase.

In this study we present a new method for site-specific labelling of antibodies by enzymatic transfer of radioactive NeuAc analogues, thus avoiding the use of oxidizing or reducing agents. Labelling of CMP-9-salizoyl-NeuAc with radioactive iodine isotopes can be rapidly performed by the iodogen method with high yields.

We are presently investigating the possibility of employing this method to label biomolecules with other radionuclides.

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