Mutational analysis of the carbohydrate-binding activity of the NeuAc(α -2,6)Gal/GalNAc-specific type 2 ribosome-inactivating protein from elderberry (*Sambucus nigra*) fruits

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Sambucus nigra agglutinin I (SNA-I) is a type 2 ribosome-inactivating protein. Site-directed mutagenesis was used to mimic the conversion of the highly active B-chain of fruit-specific SNA (SNA-If) into the completely inactive B-chain of the closely related and naturally occurring loss-of-activity mutant called *S. nigra* agglutinin lectin-related protein. In the first mutant SNA-If-M1 the high-affinity site 2 of SNA-If was disrupted by replacing the presumed critical residue Asp²³¹ with Glu²³¹. In the double mutant SNA-If-M2, site 1 of SNA-If-M1 was also disrupted by substituting the presumed critical residue Asn⁴⁸ with Ser⁴⁸. The parent type 2 ribosome-inactivating protein and both mutants were expressed in *Nicotiana tabacum* Samsun NN and

the recombinant proteins were purified and analysed. Recombinant SNA-If agglutinated rabbit erythrocytes equally well as SNA-If, but both mutants were completely inactive in this test. Binding assays to immobilized galactose and fetuin revealed that the mutation Asp²³¹ → Glu²³¹ reduces the affinity of the B-chain for galactose and fetuin by more than 50%. Furthermore, the introduction of the second mutation Asn⁴⁸ → Ser⁴⁸ reduces the binding activity to less than 20% of the original activity.

Key words: binding site, galactose, lectin, mutant, recombinant protein.

INTRODUCTION

Type 2 ribosome-inactivating proteins (RIPs) are a group of chimeric proteins built up of an A-chain and a B-chain linked by an interchain disulphide bridge. The A-chain consists of an RNA *N*-glycosylase domain (EC 3.2.2.22) catalysing the endohydrolysis of the *N*-glycosidic bond at one specific adenine of the large ribosomal RNA, whereas the B-chain comprises a typical lectin domain (see [1–3] for reviews).

Sequence analysis and structural studies demonstrated that the B-chains of type 2 RIPs consist of six tandemly arrayed subdomains of approx. 40 amino acid residues. These subdomains (numbered 1 α , 1 β , 1 γ , 2 α , 2 β and 2 γ starting from the *N*-terminus) are arranged in two domains with a typical three-lobed β -trefoil fold. Although all the six subdomains share high sequence similarity and have a similar structure, only subdomains 1 α and 2 γ possess a functional carbohydrate-binding site [3,4]. Moreover, detailed studies with ricin revealed that the binding sites associated with these two subdomains differ in affinity and specificity [5,6]. The site located in subdomain 1 α (referred to as site 1) binds only Gal and is considered as a low-affinity site, whereas the carbohydrate-binding site located in subdomain 2 γ (referred to as site 2) accommodates both Gal and GalNAc and behaves as a high-affinity site. Site-directed mutagenesis of the B-chain of ricin reveals that the replacement of a single amino acid residue abolishes or drastically reduces the carbohydrate-binding activity of a given site [7,8].

Biochemical and molecular studies revealed that the bark of elderberry expresses a complex mixture of type 2 RIPs. Two of these type 2 RIPs, called *Sambucus nigra* agglutinin I (SNA-I) [9]

and *S. nigra* agglutinin V [10], possess fully active B-chains and behave as genuine lectins. The most abundant bark protein is also a type 2 RIP closely resembling SNA-I. But it is completely devoid of sugar-binding activity and is accordingly called *S. nigra* lectin-related protein (SNLRP) [11]. Molecular modelling indicated that the lack of lectin activity is due to the replacement of a single crucial amino acid residue in both carbohydrate-binding sites. Since SNLRP can be regarded as a natural mutant of SNA-I, the SNA-I–SNLRP system offers a unique opportunity to reconstruct by site-directed mutagenesis the conversion of a fully active type 2 RIP into an inactive equivalent through a few amino acid changes in the sugar-binding sites.

The present study reports the construction and expression of two mutants of the fruit-specific SNA-I (SNA-If) in tobacco (*Nicotiana tabacum* Samsun NN) plants. Carbohydrate-binding site 2 was mutated (Asp²³¹ → Glu²³¹) in the first mutant (SNA-If-M1), whereas sites 1 (Asn⁴⁸ → Ser⁴⁸) and 2 (Asp²³¹ → Glu²³¹) were mutated in the double mutant (SNA-If-M2). The recombinant wild-type and mutant proteins were purified from tobacco and compared with native SNA-If from elderberry fruits. Specifically, their molecular structure, RNA *N*-glycosylase activity and carbohydrate-binding properties were studied.

EXPERIMENTAL

Materials

A cDNA encoding SNA-If was previously isolated from a cDNA library constructed with poly(A)⁺RNA (polyadenylated RNA) from elderberry fruits (GenBank[®] accession no. AF012899). The

Abbreviations used: pGB19, plant transformation vector; RIP, ribosome-inactivating protein; SNA-I, *Sambucus nigra* agglutinin I; rSNA-I, recombinant SNA-I; SNA-If, fruit-specific SNA; SNA-If-M1, SNA-If mutated in carbohydrate-binding site 2 (Asp²³¹ → Glu²³¹); SNA-If-M2, SNA-If mutated in carbohydrate-binding sites 1 (Asn⁴⁸ → Ser⁴⁸) and 2 (Asp²³¹ → Glu²³¹); SNLRP, *Sambucus nigra* lectin-related protein.

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plant transformation vector (pGB19) was constructed by transfer of the *EcoRI*–*HindIII* fragment of the plasmid pFF19 [containing the cauliflower mosaic virus enhancer (duplicated), promoter and polyadenylation signal] [12] into pGPTV-BAR [13] from which the β -glucuronidase gene was removed by *EcoRI*–*HindIII* digestion. The vector pGB19 still contains the phosphinothricin acetyl transferase (*bar*) gene conferring phosphinothricin resistance.

Mutagenesis and construction of recombination vectors

All plasmids were constructed by standard cloning and PCR techniques [14]. The complete coding sequence of SNA-If was amplified by PCR using cDNA encoding SNA-If as the template. The primers used were such that the generated SNA-If sequence was flanked by a 5'-end *XbaI* site and a 3'-end *SacI* site. Mutations in the SNA-If B-chain were generated by overlap PCR using mutagenic oligonucleotides designed in such a way that mismatches were flanked on either side by at least 25 bp complementary to the wild-type sequence. In the first mutant SNA-If-M1, amino acid Asp²³¹ (codon GAC) in the B-chain of SNA-If was replaced by Glu (codon GAA). An additional mutation whereby Asn⁴⁸ (codon AAC) of SNA-If-M1 was replaced by a Ser (codon AGC) residue yielded the double mutant SNA-If-M2.

The restricted fragments (1.8 kb) were subcloned into the *SacI* and *XbaI* sites of the plasmid pFF19. After confirmation of the sequence of the constructs by dideoxy sequencing [15], the plasmids were digested with *SacI* and *XbaI* and the inserts were cloned into the vector pGB19. The resulting plasmids were designated as pGB19–SNA-If, pGB19–SNA-If-M1 and pGB19–SNA-If-M2 respectively.

Transformation of tobacco

Agrobacterium tumefaciens GV3101 was transformed with the plasmid pGB19, which contains the sequences encoding SNA-If, SNA-If-M1 or SNA-If-M2 by electroporation. The *Agrobacterium* strains containing the different pGB19 constructs were used for transformation of tobacco leaf discs by the method of Rogers et al. [16]. Shoots were selected on the Murashige and Skoog [17] medium containing 0.1 μ g/ml α -naphthalene acetic acid, 1 μ g/ml 6-benzylaminopurine, 100 μ g/ml timentin, 100 μ g/ml cefotaxime, 100 μ g/ml carbenicillin and 5 μ g/ml phosphinothricin. Resistant shoots were transferred for rooting to Murashige and Skoog medium containing 0.1 μ g/ml α -naphthalene acetic acid, 100 μ g/ml timentin, 100 μ g/ml cefotaxime, 100 μ g/ml carbenicillin and 5 μ g/ml phosphinothricin. Tobacco plants were then transferred to soil and grown in a greenhouse under 16 h light cycles (55% humidity and day/night temperature of 20/18 °C).

RNA isolation and Northern-blot analysis

RNA was prepared from tobacco leaves as described by Eggermont et al. [18] with a minor modification. Total RNA was precipitated by adding 0.5 vol. of 8 M lithium chloride and incubating overnight at –20 °C. After centrifugation, RNA was dissolved in RNase-free water and quantified spectrophotometrically.

RNA electrophoresis was performed as described by Sambrook et al. [14]. Approximately 30 μ g of total RNA was denatured in glyoxal and DMSO, and separated in a 1.2% (w/v) agarose gel. Following electrophoresis, the RNA was transferred to an Immobilon N membrane (Millipore) and the blot hybridized by using a random-primer-labelled cDNA insert or a specific

oligonucleotide probe for SNA-If [19]. An RNA ladder (0.16–1.77 kb) was used as a marker.

Protein extraction and Western-blot analysis

Samples (200 mg) of tobacco leaves were homogenized in 1 ml of 50 mM acetic acid by using a Fastprep system and centrifuged at 13000 *g* for 5 min. Under these conditions, most proteins are precipitated but the acid-stable type 2 RIPs remain in solution. Aliquots (200 μ l) of the cleared extracts were freeze-dried and dissolved in 20 μ l of loading buffer (0.1 M Tris/HCl, pH 7.8/4% SDS/10% glycerol containing 0.1 M β -mercaptoethanol). Samples (15 μ l each) were analysed by SDS/PAGE (15% gel) using a discontinuous system as described by Laemmli [20]. After electrophoresis, proteins were transferred to an Immobilon P membrane (Millipore) by using a semi-dry blotting system. Immunodetection was performed as described by Desmyter et al. [21], using an affinity-purified polyclonal rabbit antibody raised against SNA-I from elderberry bark as the primary antibody.

Purification of recombinant proteins

Leaves of transgenic plants were harvested and homogenized in a solution of 1 g/l ascorbic acid. After centrifugation at 4000 *g* for 10 min, 1 g/l CaCl₂ was added to the supernatant and the pH adjusted to 9.0. The extract was cleared by centrifuging at 8000 *g* for 10 min and filtered through Whatman 3MM filter paper. The clear filtrate was adjusted to pH 2.8 with 0.5 M HCl. The filtrate was loaded on to a column (2.6 cm $\times$ 10 cm; approx. 50 ml bed volume) of S Fast Flow (Amersham Pharmacia Biotech, Uppsala, Sweden) equilibrated with 20 mM acetic acid. After loading, the column was washed with 25 mM sodium formate (pH 3.8) and the bound proteins were eluted with the same buffer containing 0.3 M NaCl.

For the purification of recombinant wild-type SNA-If, the protein fraction desorbed from the S Fast Flow column was adjusted to pH 7.0 with 0.5 M NaOH and loaded on to a column (1.6 cm $\times$ 5 cm; approx. 10 ml bed volume) of fetuin–Sephacrose 4B. After passing the partially purified protein fraction, the column was washed with 0.2 M NaCl until the A_{280} decreased below 0.01 and the bound lectin was desorbed with 20 mM acetic acid. The affinity-purified lectin was dialysed against appropriate buffers and stored at –20 °C until use.

For the purification of the mutant lectins, the partially purified protein fraction was concentrated by freeze-drying and loaded on to a column (2.6 cm $\times$ 70 cm; approx. 350 ml bed volume) of Sephacryl 100 equilibrated with 20 mM sodium acetate (pH 5.0) containing 0.2 M NaCl. The column was eluted with the same buffer and fractions were collected. The first peak (eluted at the same position as native SNA-If) contained a single protein which yielded the same pattern as SNA-If using SDS/PAGE under non-reducing conditions (typified by the occurrence of high- M_r bands). Peak fractions were pooled, dialysed against appropriate buffers and stored in small aliquots at –20 °C until use.

RNA *N*-glycosylase activity assay

The RNA *N*-glycosylase activity of the RIPs was determined by the method of Endo et al. [22] using rabbit reticulocyte lysate and wheat germ ribosomes as the substrate. RIP and ribosome solutions were incubated in RIP buffer (25 mM Tris/HCl, pH 7.4/25 mM KCl/5 mM MgCl₂) at appropriate temperatures (37 and 25 °C for rabbit reticulocyte lysate and wheat germ ribosomes respectively). Total ribosomal RNA was isolated and treated with acidic aniline as described by Hao et al. [23]. The formation of Endo's fragments was checked after electrophoresis in a 1.2%

agarose–formamide gel. Gels were stained with ethidium bromide and photographed on a UV transilluminator.

Agglutination assays

Agglutination assays were performed in 96-unit-welled microtitre plates in a final volume of 50 μ l containing 40 μ l of a 1% suspension of trypsin-treated rabbit erythrocytes and 10 μ l of extracts or lectin solutions. To determine the specific agglutination activity, the lectin was serially diluted with 2-fold increments. Agglutination was assessed visually after 1 h at room temperature (20 °C). Rabbit erythrocytes were treated with trypsin as described previously [24].

Binding of recombinant SNA-If (rSNA-If) and mutant type 2 RIPs on immobilized galactose and fetuin

The carbohydrate-binding activity of rSNA-If and the mutant type 2 RIPs SNA-If-M1 and SNA-If-M2 was analysed by affinity chromatography on galactose–Sepharose 4B and fetuin–Sepharose 4B. The purified recombinant protein (10 mg) dissolved in 10 ml 1 M ammonium sulphate was loaded on to a column (1.5 cm $\times$ 5 cm, 10 ml bed volume) of galactose–Sepharose 4B equilibrated with 1 M ammonium sulphate. After washing the column with 20 ml of 1 M ammonium sulphate, the bound proteins were eluted with unbuffered 20 mM 1,3-diaminopropane. Fractions of 1 ml were collected and the A_{280} was measured. Affinity chromatography on a column (1.5 cm $\times$ 5 cm, 10 ml bed volume) of immobilized fetuin was performed similarly, except that the proteins were dissolved in 0.2 M NaCl, and 0.2 M NaCl was used as a running buffer.

Molecular modelling

Molecular modelling of the B-chain of SNA-If and the mutants SNA-If-M1 and SNA-If-M2 was performed on a Silicon Graphics O2 R10000 workstation, by using the programs INSIGHTII, HOMOLOGY and DISCOVER (MSI, San Diego, CA, U.S.A.). The atomic co-ordinates of ricin (RCSB Protein Data Bank code 2AAI) [4] were used to build the three-dimensional models of the B-chains. Steric conflicts, resulting from the replacement or deletion of some residues in SNA-If, were corrected during the model-building procedure by using the rotamer library [25] and the search algorithm implemented in the HOMOLOGY program [26] to maintain proper side-chain orientation. Energy minimization and relaxation of the loop regions were performed by several cycles of steepest descent and conjugate gradient using the cvff forcefield of DISCOVER. The program TURBOFRODO (Bio-Graphics, Marseille, France) was run on the O2 workstation to draw the Ramachandran plot and perform the superposition of the models. PROCHECK [27] was used to assess the geometric quality of the three-dimensional models.

Docking of galactose in the carbohydrate-binding sites of SNA-If and the mutants SNA-If-M1 and SNA-If-M2 was performed with the HOMOLOGY program. The lowest apparent binding energy E_{bind} (expressed in kcal/mol) compatible with the hydrogen bonds found in the ricin–lactose complex [4] was calculated with the cvff forcefield, taking into consideration van der Waals interactions, strong hydrogen bonds [$2.5 \text{ \AA} < \text{dist}(\text{D}-\text{A}) < 3.1 \text{ \AA}$ and $120^\circ < \text{ang}(\text{D}-\text{H}-\text{A}) < 180^\circ$] and weak hydrogen bonds [$2.5 \text{ \AA} < \text{dist}(\text{D}-\text{A}) < 3.5 \text{ \AA}$ and $105^\circ < \text{ang}(\text{D}-\text{H}-\text{A}) < 120^\circ$], where D is the donor, A the acceptor and H the hydrogen atom. The E_{bind} was used to anchor the pyranose ring of Gal to the binding sites of subdomains 1 α and 2 γ . To prevent any steric clash, the most compatible conformers of the modified Ser⁴⁸

(carbohydrate-binding site 1) and Glu²³¹ residues (carbohydrate-binding site 2) were used for the calculations.

RESULTS AND DISCUSSION

Identification of the amino acids essential for the carbohydrate-binding activity of SNA-If

SNLRP can be considered a natural loss-of-activity mutant of the NeuAc(α -2,6)Gal/GalNAc-binding SNA-I/SNA-If, which allows identification of the amino acid substitutions that lead to inactivation of the carbohydrate-binding sites. Alignment of the core sequences of the subdomain 1 α reveals that Asn⁴⁸ of SNA-If (Asn⁴⁶ of ricin) is replaced by Ser⁴⁵ in SNLRP. In addition, Gly⁴¹ of SNA-If (Trp³⁷ of ricin) is replaced by Leu³⁸ in SNLRP (Figure 1). Taking into account the fact that the latter amino acid is quite variable among different type 2 RIP sequences, we assume that the amino acid substitution at position 48 of the SNA-If B-chain is more important for the inactivation of the carbohydrate-binding site 1 of SNLRP. In the carbohydrate-binding site 2, only Asp²³¹ of the SNA-If B-chain (similar to Asp²³⁴ of ricin) is replaced by Glu²³⁰ in SNLRP (Figure 1). This suggests that Asp²³¹ plays an important role in the conformation of the site and the binding to carbohydrates. To check the contribution of residues Asn⁴⁸ and Asp²³¹ to the carbohydrate-binding activity of the B-chain of SNA-If, an attempt was made to mimic the apparent deleterious effect of the amino acid replacements at positions 48 and 231 in the B-chain of SNLRP by site-directed mutagenesis of SNA-If.

Design of the mutants

The single-mutant SNA-If-M1 was constructed by replacing Asp²³¹ of the SNA-If B-chain by a Glu residue. This amino acid substitution mimics the conversion of the active (high-affinity) site 2 of SNA-If into the inactive site 2 of SNLRP, but does not dramatically change the overall conformation of site 2. Molecular modelling showed that the most favourable conformer of Glu²³¹ forms two hydrogen bonds with galactose [Glu²³¹O δ 2–O3Gal (2.81 Å) and Glu²³¹O δ 1–O3Gal (2.76 Å)] equivalent to hydrogen bonds Asp²³¹O δ 2–O3Gal (2.88 Å) and Asp²³¹O δ 1–O4Gal (1.85 Å) observed in the parent SNA-If (results not shown). However, due to the unfavourable geometry of both the hydrogen bonds, one can expect fairly that the mutated site is less reactive towards Gal and related sugars than the original binding site.

The double mutant SNA-If-M2 was constructed from the single mutant SNA-If-M1 by substituting Asn⁴⁸ with Ser. This

SNA-If	1 α	CVDVRYGHIYDGNPVQLGPGCN--ECNQLW
RICIN	1 α	CVDVRDGRFHNGNAIQWLPCSKNTDAQLW
SNLRP	1 α	CVDVRDGLAKDGNPVQLLSCGQ--QSQQW
SNA-If	2 γ	AKLLMVAQRDVSRLRKIIILYRPTGNPNQWITTT
RICIN	2 γ	SGLVLVDVRASDASLKQIILYPLHGDPNQIWLPLF
SNLRP	2 γ	AKLVMEVRQSDVSLRQIIIIYHPTGNLNLQWITDT

Figure 1 Alignment of amino acid sequences of the subdomains 1 α and 2 γ of SNA-If, ricin and SNLRP

Residues forming the carbohydrate-binding sites of SNA-If, ricin [5] and SNLRP [11] are boxed in grey and the two mutated amino acids are boxed in black.

amino acid replacement mimics the conversion of the active (low-affinity) site 1 of SNA-If into the inactive site 1 of SNLRP. Molecular modelling of the double-mutant SNA-If-M2 (Asn⁴⁸ → Ser⁴⁸; Asp²³¹ → Glu²³¹) indicated that the replacement of Asn⁴⁸ in site 1 by a less extended Ser residue caused no steric clash between Ser⁴⁸ and the other surrounding residues. Docking experiments further revealed that the network of hydrogen bonds anchoring Gal to the modified binding site is very similar to that found in the parent SNA-If protein. The hydrogen bond between Asn⁴⁸Nδ2 and GalO3 (2.52 Å) is replaced by a similar hydrogen bond between Ser⁴⁸Oδ and GalO3 (3.21 Å; results not shown). However, due to the conformation of the most favourable conformer of Ser⁴⁸, the bent hydrogen bond formed between Oy of Ser⁴⁸ and O3 of Gal is weaker than the bond between Nδ2 of Asn⁴⁸ and O3 of Gal in the parent SNA-If. It can be expected therefore that site 1 of SNA-If-M2 will exhibit a much lower affinity for Gal than the corresponding site of SNA-If.

Construction and expression of rSNA-If and its mutants

Transgenic tobacco plants expressing rSNA-If or one of the mutant proteins were regenerated from plant leaf discs transformed with *A. tumefaciens* harbouring the vectors pGB19-SNA-If, pGB19-SNA-If-M1 or pGB19-SNA-If-M2. Fifteen independent phosphinothricin-resistant tobacco lines were obtained after transformation with the SNA-If construct, whereas 10 and 12 independent lines were obtained with the mutant constructs encoding SNA-If-M1 and SNA-If-M2 respectively. Seven, 4 and 6 of these transgenic lines encoding rSNA-If, SNA-If-M1 and SNA-If-M2 respectively yielded a fragment of the expected size of 1.8 kb after PCR amplification of tobacco genomic DNA using specific primers for SNA-If (results not shown).

Northern-blot analysis of the putative transformants expressing SNA-If yielded clear hybridization signals for 4 out of 7 transgenic plants (Figure 2Aa). Three out of 4 putative transgenic lines expressing SNA-If-M1 (Figure 2Ba) and all 6 transgenic lines expressing SNA-If-M2 (Figure 2Ca) reacted positively to Northern-blot analysis. RNA from untransformed tobacco yielded no signal after hybridization with a probe for SNA-If.

Western-blot analysis of crude extracts from leaves confirmed that SNA-If is exclusively expressed in transgenic, but not in non-transformed, tobacco plants (Figure 2Ab). There was also a good correlation between the presence of mRNAs encoding the mutant type 2 RIPs and the proteins (Figure 2Bb and 2Cb). None of the tobacco plants expressing SNA-If or one of the mutants showed an aberrant phenotype.

Purification and characterization of rSNA-If and its mutants

rSNA-If, SNA-If-M1 and SNA-If-M2 were purified from leaves of transgenic tobacco plants. The overall yield was approx. 9.3, 7.3 and 4.2 mg for SNA-If, SNA-If-M1 and SNA-If-M2 respectively per kg of leaves (fresh weight).

SDS/PAGE under non-reducing conditions yielded the same migration pattern as for SNA-If isolated from elderberry fruits for all three recombinant proteins. The presence in this pattern of high- M_r bands is diagnostic for SNA-If and their equivalents from other tissues. Upon reduction, the high M_r bands disappeared, giving rise to a polypeptide band of approx. 35 kDa (Figure 3). As demonstrated previously for SNA-If, this 35 kDa polypeptide band is a double band comprising both the A- and B-chains of the RIP [9]. Western-blot analysis of the purified recombinant type 2 RIPs confirmed that the polypeptides react with an antiserum directed against SNA-I from elderberry fruits. rSNA-If as well as SNA-If-M1 and SNA-If-M2 eluted at

Figure 2 Northern- and Western-blot analyses of tobacco lines transformed with constructs encoding SNA-If (A), SNA-If-M1 (B) and SNA-If-M2 (C)

(a), (b) Northern- and Western-blot analyses respectively of the transgenic tobacco lines. The lanes indicated by numbers refer to the tobacco lines transformed with the construct for SNA-If (A, lanes 1–7), SNA-If-M1 (B, lanes 1–4) and SNA-If-M2 (C, lanes 1–6). Lane WT shows the analysis for a wild-type untransformed tobacco plant. Lane P shows Western-blot analysis of purified SNA-If from elderberry fruits.

the same position as SNA-If upon gel filtration, indicating that they all adopt the same [A-s-s-B-s-s-B-s-s-A]₂ structure as SNA-If.

RNA *N*-glycosylase activity of rSNA-If, SNA-If-M1 and SNA-If-M2

The RNA *N*-glycosylase activity of rSNA-If and the two mutant proteins was determined by using ribosomes from both animal and plant origin as the substrate. All three recombinant proteins depurinated rabbit reticulocyte ribosomes, but were inactive towards wheat germ ribosomes (results not shown). Similar results were obtained with SNA-If isolated from elderberry fruits. Determination of the specific RNA *N*-glycosylase activity indicated that all three recombinant proteins were equally active as SNA-If, the lowest concentration required to depurinate rabbit reticulocyte ribosomes being 50 pM. It can be concluded therefore that the site-directed mutations in the B-chain do not result in any detectable change in the activity of the A-chain.

Figure 3 SDS/PAGE of SNA-If and recombinant proteins from transgenic tobacco

Samples (10 μ g each) of the unreduced (lanes 1–4) and reduced (lanes 5–8) proteins were loaded as follows: lanes 1 and 5, SNA-If; lanes 2 and 6, rSNA-If; lanes 3 and 7, SNA-If-M1; lanes 4 and 8, SNA-If-M2. Molecular-mass reference proteins (lane R) were lysozyme (14 kDa), soya bean trypsin inhibitor (20 kDa), carbonic anhydrase (30 kDa), ovalbumin (43 kDa), BSA (67 kDa) and phosphorylase *b* (94 kDa).

Agglutination activity of rSNA-If and its mutants

Agglutination assays with the purified proteins revealed that rSNA-If agglutinates rabbit erythrocytes as effectively as SNA-If, the minimal concentration required for agglutination being 5 μ g/ml. In contrast, neither rSNA-If-M1 nor rSNA-If-M2 showed a visible agglutination activity even when the final concentration was increased to 2 mg/ml. Hapten inhibition experiments indicated that rSNA-If and SNA-If exhibit the same sensitivity towards galactose (10 mM) and fetuin (60 μ g/ml).

Binding of rSNA-If, rSNA-If-M1, rSNA-If-M2 and SNLRP to immobilized galactose and fetuin

The failure of the mutant lectins to agglutinate rabbit erythrocytes does necessarily imply that the proteins are completely devoid of carbohydrate-binding activity, because the loss of agglutination activity may also be due to a change in affinity or specificity. It is worth mentioning in this context that several type 2 RIPs are poor lectins although they readily bind to immobilized sugars or glycoproteins. To check whether the mutant lectins still possess carbohydrate-binding activity and if so to what extent, their binding to immobilized galactose and fetuin was quantified. Purified rSNA-If, rSNA-If-M1 and rSNA-If-M2 were applied on to columns of galactose-Sepharose 4B and fetuin-Sepharose 4B, and their binding or elution were studied.

SNA-If from elderberry fruits was quantitatively retained on galactose-Sepharose 4B and fetuin-Sepharose 4B (results not shown). As could be expected on the basis of the results of the agglutination tests, rSNA-If also bound quantitatively to both the affinity columns (Figure 4). The binding of SNA-If-M1 (with a modified high-affinity site 2) was strongly decreased as compared with that of rSNA-If. Only 48 and 43 % of the protein was retained, indeed, on the immobilized galactose and fetuin respectively. A similar decrease in binding activity was obtained with a mutant of the ricin B-chain (Asp²³⁴ $\rightarrow$ Glu²³⁴; Ala²³⁷ $\rightarrow$ Arg²³⁷; partly equivalent to the Asp²³¹ $\rightarrow$ Glu²³¹ mutation introduced in SNA-If-M1), which exhibited a reduced affinity for both asialofetuin and KB mammalian cells [28].

Figure 4 Binding of the rSNA-If, SNA-If-M1 and SNA-If-M2 to (A) galactose-Sepharose 4B and (B) fetuin-Sepharose 4B

Approximately 10 mg of the RIP was loaded on to the column. After washing, bound proteins were eluted with 20 mM diaminopropane. Fractions of 1 ml were collected and the A_{280} was determined. $\times$, SNA-If; $\blacksquare$, SNA-If-M1; $\blacktriangle$, SNA-If-M2; $\bullet$, SNLRP.

As predicted by the molecular modelling studies, the carbohydrate-binding activity of the double mutant was even more affected. Only 20 and 17 % of SNA-If-M2 was retained on galactose-Sepharose 4B and fetuin-Sepharose 4B respectively. The results of the site-directed mutagenesis leave no doubt that Asn⁴⁸ and Asp²³¹ largely determine the activity of sites 1 and 2 respectively of the SNA-If B-chain. However, it should be emphasized that even the double mutant possesses a residual sugar-binding activity, whereas SNLRP exhibited no activity at all in the same assays. This implies that the two replacements Asn⁴⁸ $\rightarrow$ Ser⁴⁸ and Asp²³¹ $\rightarrow$ Glu²³¹ are not sufficient to explain the conversion of the fully active B-chain of SNA-If into the completely inactive equivalent of SNLRP. Most probably, other amino acid replacements introduced into SNLRP are responsible for the loss of the residual activity present in SNA-If-M2 and SNA-If-M1. It is important therefore to realize that site-directed mutagenesis has its limits and that additional information from naturally occurring loss-of-function mutants can be very helpful.

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