Choice of peptide and peptide length for the generation of antibodies reactive with the intact protein

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Choice of peptide and peptide length for the generation of antibodies reactive with the intact protein

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N-terminal peptides of bovine ribonuclease (RNase) of 20, 13 and 7 amino acid residues were isolated by reversed-phase high-performance liquid chromatography (HPLC). Antibodies were raised in mice against these peptides coupled to bovine serum albumin (BSA). It was shown that antibodies against the peptides reacted with the intact protein and that the immune response decreased with decreasing size of peptide. In order to obtain a satisfactory reaction with the intact protein, the peptide immunogen should be longer than 7 amino acids.

Antipeptide antibody *Ribonuclease* *Secondary structure* *Immunization*

1. INTRODUCTION

There is a rapidly growing interest in antibodies elicited against peptides which react with the intact protein [1]. It has been shown that the generation of such antibodies is a high-frequency event [2] which cannot easily be rationalized if a random conformation is assumed for small peptides in solution. Here, the importance of peptide length for the generation of such antibodies is investigated by using peptide fragments of a protein (bovine ribonuclease) of which the amino acid sequence [3] and the tertiary structure [4,5] are known.

2. MATERIALS AND METHODS

2.1. *Isolation of peptide fragments*

Peptide 1–20 was obtained by limited proteolysis of bovine ribonuclease (Miles-Seravac, Maidenhead, England) with subtilopeptidase A (Fluka AG, Buchs, Switzerland) as described [6].

Abbreviations: RNase, bovine ribonuclease; HPLC, high-performance liquid chromatography; BSA, bovine serum albumin

Peptide 1–13 was obtained by CNBr-treatment of RNase. RNase (10 mg/ml) in 70% formic acid was treated with a 100-fold molar excess (with respect to the 4 methionines in RNase) of CNBr (Fluka) for 19 h at room temperature. The reaction mixture was diluted with water and lyophilized. Peptide 1–13 was obtained by gel filtration on a Sephadex G-25 (fine) column (157 × 1 cm) in 0.2 M acetic acid at a flow-rate of 7 ml/h. The absorbance was monitored at 230 nm and fractions (1.2 ml) were analyzed by high-voltage paper electrophoresis at pH 3.5 [7].

To obtain peptide 1–7, performic acid-oxidized RNase [8] was digested with trypsin TPCK (Millipore, Freehold, NJ, USA) in 0.1 M ammoniumbicarbonate (pH 8.2) at an E/S ratio of 1:100 (w/w) for 4 h at 37°C. The reaction was terminated by lyophilization. The peptide mixture was fractionated by gel filtration and analyzed as described above for peptide 1–13. The fractions containing peptide 1–7 were further purified by preparative high-voltage paper electrophoresis [7].

2.2. *Further purification and amino acid analysis*

Peptides were further purified by reversed-phase HPLC on a column (30 × 0.46 cm) of Nucleosil 10

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C-18 (Macherey-Nagel, Duren, FRG) which was eluted with a linear gradient of acetonitrile in 0.1% trifluoroacetic acid at a flow-rate of 1 ml/min. The absorbance was monitored at 214 nm.

Amino acid analysis was performed with a Kontron Liquimat III amino acid analyzer. Samples were hydrolyzed as described [7].

2.3. *Preparation of peptide conjugates and immunization*

Peptdies were coupled to BSA with glutaraldehyde [9]. The percentage coupling was determined by amino acid analysis.

C57Bl mice (3–4 per group) were immunized by intraperitoneal injection of 0.1 ml of peptide conjugate diluted with an equal volume of Freunds complete adjuvant. A booster injection was given 3 weeks later. Mice were bled after 5 weeks and the serum was collected. Each mouse received approx. 50 μg BSA with 7 μg peptide per injection. Control groups received either RNase (10 μg per injection) or BSA (50 μg per injection).

The immune response was determined by inactivation of a bacteriophage T4-RNase conjugate [10]. RNase was coupled to phage T4 with glutaraldehyde. Antiserum dilutions were incubated with a suitable dilution of the phage T4-conjugate at 37°C for 2 h. The mixtures were plated with *Escherichia coli B* and incubated overnight at 37°C. The % immune response was calculated from the degree of inactivation. The immune response obtained with antiserum from mice immunized with RNase was taken as 100% (minimal number of plaques) and the response obtained with antiserum against BSA was taken as 0% (maximal number of plaques). The % immune response obtained by immunization with the peptide-conjugates was determined with 10-, 25- and 50-times diluted antiserum from the number of plaques.

3. RESULTS AND DISCUSSION

Fig.1 shows the location of the HPLC-purified peptides in the structure of ribonuclease. Table 1 shows their amino acid composition. The % coupling of peptides to BSA was more than 90%. The immune response obtained by immunization with the peptide-BSA conjugates was investigated in individual mice and the results are shown in fig.2. The immune response varies considerably in individual mice immunized with the same peptide but is highest in mice immunized with peptide 1–20 and lowest after immunization with the 1–7 peptide.

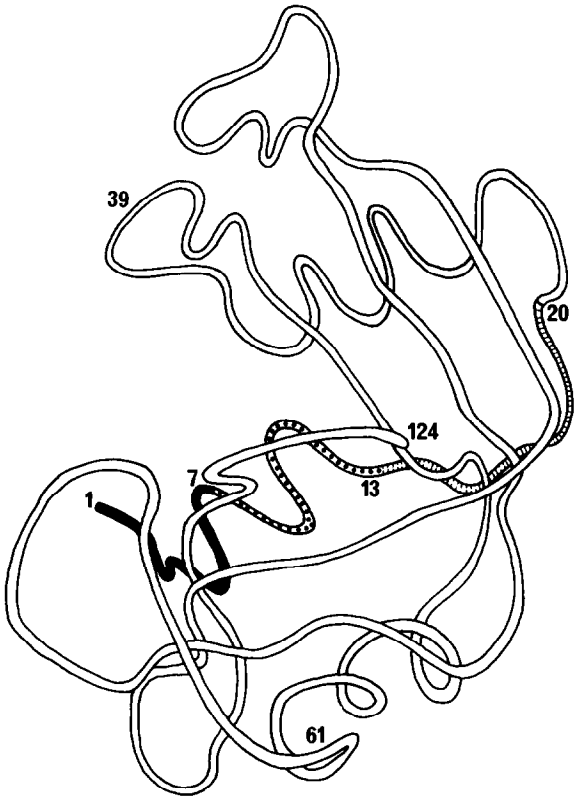

Fig.1. The location of the tryptic peptide 1–7, the 1–13 fragment obtained by cyanogen bromide treatment and the 1–20 peptide resulting from subtilisin digestion in the structure of bovine pancreatic ribonuclease.

The finding that antibodies to a stretch of consecutive amino acids of a protein, i.e., a peptide, may react with the intact protein has opened new fields of investigation [1,2]. Surprisingly, most antibodies reacted far better than expected with the native protein [2]. One of the explanations was that the conformation of the peptide in solution might be less random than expected from physicochemical studies. A recent study [11] shows that the conformation of a 21-residue C-terminal peptide is random in water but helical in a more hydrophobic environment. This suggests that the inherent potential of a peptide to exist in a particular secondary structure might be positively influenced by its environment, i.e., coupling the pep-

Table 1

Amino acid composition of peptides 1–20, 1–13 and 1–7

	Peptide		
	1–20	1–13	1–7
Asp	1.2 (1)		
Thr	2.0 (2)	1.0 (1)	0.9 (1)
Ser	3.0 (3)		
Hser + Hserlact		0.9 (1)	
Glu	3.1 (3)	3.3 (3)	1.0 (1)
Ala	5.0 (5)	3.1 (3)	3.0 (3)
Met	1.1 (1)		
Phe	0.9 (1)	1.0 (1)	
Lys	2.3 (2)	1.8 (2)	2.0 (2)
His	1.0 (1)	1.0 (1)	
Arg	1.0 (1)	1.0 (1)	

Methionine is converted to homoserine and homoserinelactone. The expected composition of the peptides derived from the amino acid sequence (see below) is shown in parentheses. Lys-Glu-Thr-Ala-Ala-Ala-Lys-Phe-Glu-Arg-Gln-His-Met-Asp-Ser-Ser-Thr-Ser-Ala-Ala

tide to a protein carrier or during the process of eliciting antibodies. It might be argued that a longer peptide will facilitate the folding to a secondary structure which is also present in the native protein. Therefore, it is plausible that immunization with peptides longer than 7 amino acids will result in a higher immune response and a better reaction of antipeptide antibodies with the intact protein than smaller peptides which have less possibilities to exist in an adequate immunogenic structure. In addition, the possibility that a longer peptide comprises more than one antigenically important region may play a role. Shapira et al. [12] synthesized 8-, 18- and 27-residue peptides corresponding to the amino acid sequence of H3 influenza hemagglutinin. Immunization with the 18- and 27-residue peptides resulted in antibodies which reacted with the intact virus, while antibodies against the 8-residue peptide did not bind the intact virus. These results suggest that peptides should be longer that 8 amino acids in order to obtain antibodies capable of reaction with the intact viral protein.

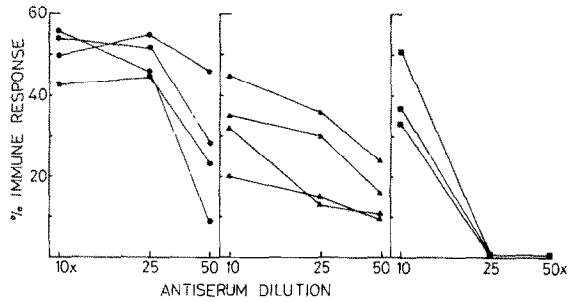

Fig.2. Immune response of mice immunized with RNase peptides conjugated to BSA. ●, Peptide 1–20; ▲, peptide 1–13; ■, peptide 1–7. The lines represent immune responses of individual mice.

Recently, it was shown that there is a good correlation between the temperature factors of the main chain atoms and antigenicity [13]. The temperature factors for the main chain atoms, i.e., the mobility of the N-terminal region of RNase [5], show (fig.3) that there is a good agreement with the accessibility of the side-chains as calculated by Lee and Richards [14]. Although a considerable amount of the antigenic regions will not be present in a linear amino acid sequence, flexible and accessible parts of a protein might be the first choice for peptide synthesis.

Therefore, it is concluded that peptides of more than 7 amino acids which are located in a flexible and accessible region of the intact protein are most suitable for the production of antipeptide antibodies which will react with the intact protein. Very often the N- or C-terminal region of a protein will meet these criteria.

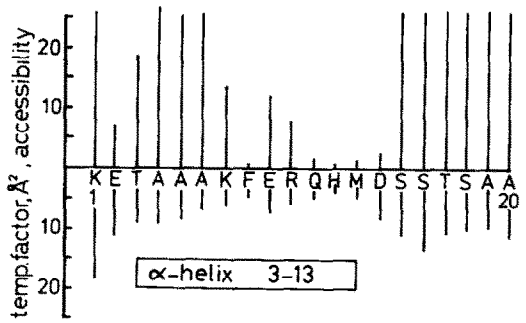

Fig.3. Side-chain accessibility (top) as determined by Lee and Richards [14] for RNase S. From the X-ray structure of RNase A [5] maximal accessibility was assumed for amino acids 15–20. The main chain temperature factors (bottom) are from [5]. The amino acid sequence of the N-terminal region of RNase is shown in the one-letter code and the alpha-helix is also indicated.

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