Pure & Appl. Chem., Vol. 66, Nos 10/11, pp. 2015–2022, 1994.
Printed in Great Britain.

Design and development of synthetic peptide antigens

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Abstract: Using hydrophilicity, surface probability, backbone flexibility and secondary structure parameters, antigenic sites of ß-thymosins (Tß), ubiquitin and gp41 envelope glycoprotein fragments of HIV-1 are determined. Corresponding antigenic sites of $Tß_4$, $Tß_9$, ubiquitin and gp41 (583-623) are produced either by tryptic cleavage from the natural peptides or by solid phase peptide synthesis (Fmoc/Bu^t strategy, Ecosyn P batch synthesizer, Eppendorf/Biotronik, Maintal, Germany). The predicted antigenic fragments allow the generation of specific antibodies for the determination of body fluid or tissue levels and immunostaining experiments, or, in the case of gp41-HIV fragments, the antigens can be used directly for the diagnosis of AIDS patients.

INTRODUCTION

In recent years it has been schown that ß-thymosins are widely distributed in numerous tissues, and different peptides have been isolated from many species of the animal kingdom ranging from sea urchin to mammals (1-3). Usually, two highly homologous ß-thymosins are present in one species. In mammals, the main ß-thymosin is $Tß_4$ which is accompanied by $Tß_{10}$ (human, horse, cat, rabbit and mouse), $Tß_9$ (bovine), $Tß_9^{Met}$ (pig) or $Tß_{13}$ (whale). In rabbit and frog tissue two peptides with slight deviations from the $Tß_4$ structure were found. $Tß_4^{Ala}$ in rabbits differs only in the first amino acid (Ala instead of Ser). From oocytes of Xenopus laevis a $ß_4$-like peptide was isolated, namely $Tß_4^{Xen}$, the primary structure of which is different in three amino acids from $Tß_4$. No ß-thymosins could be detected in invertebrates like gypsy moth larvae, the earth-worm, the meal-worm as well as in yeast (Fig. 1). As mentioned before, from the ß-thymosin family, $Tß_4$ (43 amino acid residues, mw 4982 D) is a major peptide in different cells of human, calf, rat or mouse (4-8). Its structure was confirmed by two different synthetic approaches, an automated solid phase procedure (9) and a synthesis in solution (10). Recently it has been shown that the peptide is widely distributed in numerous tissues (7,8,11). $Tß_4$ has been reported to induce terminal deoxynucleotidyl transferase activity in vivo and in vitro (12), inhibit the migration of guinea pig peritoneal macrophages (13), stimulate the hypothalamic secretion of leutinizing hormone-releasing hormone (14) or induce phenotypic changes in the Molt-4 leukemic cell line (15). Actually, $Tß_4$ has been raising a lot of scientific interest since Safer et al. (16) observed that it forms a 1:1 complex with muscle G-actin under physiological ionic conditions and thus prevents its polymerization to F-actin. Therefore, $Tß_4$ seems to be involved in the microfilament system, acting as an actin-sequestering peptide like gelsolin or profilin. Lenfant et al. (17) reported that the tetrapeptide Ac-Ser-Asp-Lys-Pro-OH, isolated from fetal calf bone marrow, shows a high inhibitory activity on the proliferation of hematopoietic pluripotent stem cells. The tetrapeptide is identical with the aminoterminal part of $Tß_4$, and it may well

be that the polypeptide is a precursor of this recently discovered inhibitor.

	5	10	15
$T\beta_4$	Ac-Ser-Asp-Lys-Pro-Asp-Met-Ala-Glu-Ile-Glu-Lys-Phe-Asp-Lys-Ser-		
$T\beta_4^{Ala}$	Ac-*Ala*-Asp-Lys-Pro-Asp-Met-Ala-Glu-Ile-Glu-Lys-Phe-Asp-Lys-Ser-		
$T\beta_4^{Xen}$	Ac-Ser-Asp-Lys-Pro-Asp-Met-Ala-Glu-Ile-Glu-Lys-Phe-Asp-Lys-*Ala*-		
$T\beta_9$	Ac-*Ala*-Asp-Lys-Pro-Asp-*Leu*-*Gly*-Glu-Ile-*Asn*-*Ser*-Phe-Asp-Lys-*Ala*-		
$T\beta_9^{Met}$	Ac-*Ala*-Asp-Lys-Pro-Asp-Met-*Gly*-Glu-Ile-*Asn*-*Ser*-Phe-Asp-Lys-*Ala*-		
$T\beta_{10}$	Ac-*Ala*-Asp-Lys-Pro-Asp-Met-*Gly*-Glu-Ile-*Ala*-*Ser*-Phe-Asp-Lys-*Ala*-		
$T\beta_{11}$	Ac-Ser-Asp-Lys-Pro-*Asn*-*Leu*-*Glu*-Glu-*Val*-*Ala*-*Ser*-Phe-Asp-Lys-*Thr*-		
$T\beta_{12}$	Ac-Ser-Asp-Lys-Pro-Asp-*Leu*-Ala-Glu-*Val*-*Ser*-*Asn*-Phe-Asp-Lys-*Thr*-		
$T\beta_{12}^{perch}$	Ac-Ser-Asp-Lys-Pro-Asp-*Ile*-*Ser*-Glu-*Val*-*Thr*-*Ser*-Phe-Asp-Lys-*Thr*-		
$T\beta_{13}$	Ac-*Ala*-Asp-Lys-Pro-Asp-Met-*Gly*-Glu-Ile-*Ala*-*Ser*-Phe-Asp-Lys-*Ala*-		
$T\beta_{14}$	Ac-*Ser*-ASp-Lys-Pro-Asp-*Ile*-*Ser*-Glu-*Val*-*Ser*-*Ser*-Phe-Asp-Lys-*Thr*-		

	20	25	30
$T\beta_4$	Lys-Leu-Lys-Lys-Thr-Glu-Thr-Gln-Glu-Lys-Asn-Pro-Leu-Pro-Ser-		
$T\beta_4^{Ala}$	Lys-Leu-Lys-Lys-Thr-Glu-Thr-Gln-Glu-Lys-Asn-Pro-Leu-Pro-Ser-		
$T\beta_4^{Xen}$	Lys-Leu-Lys-Lys-Thr-Glu-Thr-Gln-Glu-Lys-Asn-Pro-Leu-Pro-Ser-		
$T\beta_9$	Lys-Leu-Lys-Lys-Thr-Glu-Thr-Gln-Glu-Lys-Asn-*Thr*-Leu-Pro-*Thr*-		
$T\beta_9^{Met}$	Lys-Leu-Lys-Lys-Thr-Glu-Thr-Gln-Glu-Lys-Asn-*Thr*-Leu-Pro-*Thr*-		
$T\beta_{10}$	Lys-Leu-Lys-Lys-Thr-Glu-Thr-Gln-Glu-Lys-Asn-*Thr*-Leu-Pro-*Thr*-		
$T\beta_{11}$	Lys-Leu-Lys-Lys-Thr-Glu-Thr-Gln-Glu-Lys-Asn-Pro-Leu-Pro-*Thr*-		
$T\beta_{12}$	Lys-Leu-Lys-Lys-Thr-Glu-Thr-Gln-Glu-Lys-Asn-Pro-Leu-Pro-*Thr*-		
$T\beta_{12}^{perch}$	Lys-Leu-Lys-Lys-Thr-Glu-Thr-Gln-Glu-Lys-Asn-Pro-Leu-Pro-Ser-		
$T\beta_{13}$	Lys-Leu-Lys-Lys-Thr-Glu-Thr-Gln-Glu-Lys-Asn-*Thr*-Leu-Pro-*Thr*-		
$T\beta_{14}$	Lys-Leu-Lys-Lys-Thr-Glu-Thr-*Ala*-Glu-Lys-Asn-*Thr*-Leu-Pro-*Thr*-		

	35	40	
$T\beta_4$	Lys-Glu-Thr-Ile-Glu-Gln-Glu-Lys-Gln-Ala-Gly-Glu-Ser-OH		
$T\beta_4^{Ala}$	Lys-Glu-Thr-Ile-Glu-Gln-Glu-Lys-Gln-Ala-Gly-Glu-Ser-OH		2%
$T\beta_4^{Xen}$	Lys-Glu-Thr-Ile-Glu-Gln-Glu-Lys-Gln-*Thr*-*Ser*-Glu-Ser-OH		7%
$T\beta_9$	Lys-Glu-thr-Ile-Glu-gln-Glu-Lys-Gln-Ala-*Lys*-OH		22%
$T\beta_9^{Met}$	Lys-Glu-thr-Ile-Glu-Gln-Glu-Lys-Gln-Ala-*Lys*-OH		20%
$T\beta_{10}$	Lys-Glu-Thr-Ile-Glu-Gln-Glu-Lys-*Arg*-*Ser*-*Glu*-*Ile*-Ser-OH		26%
$T\beta_{11}$	Lys-Glu-Thr-Ile-Glu-Gln-Glu-Lys-Gln-Ala-*Ser*-OH		22%
$T\beta_{12}$	Lys-Glu-Thr-Ile-Glu-Gln-Glu-Lys-Gln-Ala-*Thr*-*Ala*-OH		19%
$T\beta_{12}^{perch}$	Lys-Glu-Thr-Ile-Glu-Gln-Glu-Lys-*Ala*-Ala-*Ala*-*Thr*-*Ser*-OH		21%
$T\beta_{13}$	Lys-Glu-Thr-Ile-Glu-Gln-Glu-Lys-Gln-Ala-*Lys*-OH		20%
$T\beta_{14}$	Lys-Glu-Thr-Ile-Glu-Gln-Glu-Lys-*Thr*-*Ala*-OH		29%

Fig. 1 Amino acid sequences of ß-thymosins and their deviation in % from the $T\beta_4$ primary structure.

Ubiquitin is a small protein believed to be universally present in living cells (18). It consists of a single 8565 D polypeptide chain of 76 amino acids. Ubiquitin has been isolated and sequenced from a variety of sources. Insect (19), trout (20), bovine (21) and human (22) ubiquitins have identical primary structures through arginine-74. In the original sequencing studies of bovine and human ubiquitin, arginine-74 was determined as the COOH-terminal residue. However, subsequent studies indicated that ubiquitin has 76 amino acids and terminates with Gly-Gly (23,24; Fig. 2).

H-Met-Gln-Ile-Phe-Val[5]-Lys-Thr-Leu-Thr-Gly[10]-Lys-Thr-Ile-Thr-Leu[15]-
Glu-Val-Glu-Pro-Ser[20]-Asp-Thr-Ile-Glu-Asn[25]-Val-Lys-Ala-Lys-Ile[30]-
Gln-Asp-Lys-Glu-Gly[35]-Ile-Pro-Pro-Asp-Gln[40]-Gln-Arg-Leu-Ile-Phe[45]-
Ala-Gly-Lys-Gln-Leu[50]-Glu-Asp-Gly-Arg-Thr[55]-Leu-Ser-Asp-Tyr-Asn[60]-
Ile-Gln-Lys-Glu-Ser[65]-Thr-Leu-His-Leu-Val[70]-Leu-Arg-Leu-Arg-Gly[75]-
Gly-OH

Fig. 2 Primary structure of human and bovine ubiquitin.

The 76-amino acid polypeptide is the physiologically active form (24,25), ubiquitin lacking the COOH-terminal Gly-Gly is most likely an *in vitro* proteolytic artifact. Apart from its physiological roles, ubiquitin is of interest because of its stability and unparalleled sequence conservation (19-22). It is noteworthy that the molecule is extremely resistent to tryptic digestion despite the presence of seven lysine and four arginine residues (21). It is also quite stable over a wide range of pH and temperature values. Estimations of its secondary structure have generally emphasized a highly globular compact conformation with a low percentage of α-helix and ß-sheet (26,27). In the crystal structure, a single α-helix with 16% of the residues is observed, the ß-sheet corresponds to 37% of the chain, and the seven reverse turns represent 37% of the chain. Thus, about 90% of the polypeptide chain is involved in the hydrogen-bonded secondary structure (28).

It could be shown that the major immunodominant region which is highly conserved among the HIV-1 strains, sequenced so far, is located on the transmembrane glycoprotein gp41 env. Several synthetic peptides, varying in length and/or position , but all corresponding to this region can be used as antigens in an ELISA (29,30). The main binding site was established as a 7 amino acid residue peptide reaching from amino acid 603 to 609 on the env reading frame (31-33), but it could be shown that at least one more epitope is located near this site (34). For diagnostic purposes it is essential to use a peptide antigen which covers the whole immunodomonant region, such as the fragment 583-623 on the env reading frame of the HIV-1 isolate BRU (35).

A computer program, developed by Jameson and Wolf (36), integrating hydrophilicity (H), surface probability (S), backbone flexibility (F) and secondary structure (CF: Chou-Fasman; GR: Garnier-Robson) parameters and based on the following equation

$$A_i = \sum_{i=1}^{N} 0.3\,(H) + 0.15\,(S) + 0.15\,(F) + 0.2\,(CF) + 0.2\,(GR) \qquad \text{(equ.1)}$$

is used to predict potential antigenic sites of $T\beta_4$, $T\beta_9$, ubiquitin and the transmembrane glycoprotein gp41 env fragment 583-623.

RESULTS AND DISCUSSION

Thymosin β_9 and β_4

Antibodies against $T\beta_4$ have been raised by several groups immunizing rabbits with conjugates of $T\beta_4$ and keyhole limpet hemocyanine (37-41). However, these antibodies have not been tested for cross-reactivity with other members of the $T\beta_4$ family. Such antibodies have been used for measurements of the $T\beta_4$ level in human serum and plasma (39) without taking into account their cross-reactivity for instance with $T\beta_{10}$. Our aim was to obtain specific antibodies against one member of the $T\beta_4$ family, eg. $T\beta_9$, which do not cross-react with $T\beta_4$ that is present in the same species. Antibodies of that kind should be also suitable for immunohistochemical studies. By comparing the primary sequences of $T\beta_4$ and $T\beta_9$ (Fig. 1) it becomes obvious that, though both peptides are highly homologous (78% homology), they differ most in their N-termini. According to the Chou-Fasman prediction, $T\beta_9$ should adopt a turn structure at the N-terminus and two helical regions ranging from amino acid residues 13-24 and 30-41. $T\beta_4$ has three separate helical regions and an increased number of ß-turns as predicted for $T\beta_4$ in comparison to $T\beta_9$. Both molecules, however, have high side chain solvent accessibility (42,43). According to Hopp and Woods (44), antigenic sites may be also predicted from the determination of the hydrophilicity segments of a polypeptide or protein. The Chou-Fasman plot of $T\beta_9$ with marked hydrophilicity partameters characterizes it as a very hydrophilic molecule, and, indeed, the natural (45) as well as the synthetic (46) peptide are extremely soluble in water. Also, flexible regions of a polypeptide often can be correlated

with antigenic determinants, as mobile peptide segments are most probably located on the surface of a protein. The Chou-Fasman plot with marked $T\beta_9$ flexibility parameters demonstrates that a large portion of the $T\beta_9$ molecule is predicted to have high chain flexibility. As none of the parameters, discussed before, is sufficiently predictive concerning the localization of antigenic sites in proteins, Jameson and Wolf (36) developed a computer program to predict potential antigenic determinants directly from the primary amino acid sequences as given in equation 1. In Fig.3, the predicted antigenic regions of $T\beta_9$ are marked by octagons in the Chou-Fasman plot. According to these theoretical considerations an N-terminal fragment of $T\beta_9$ should be most suitable for the development of specific antibodies showing no cross-reactivity with other members of the ß-thymosin family, and such a segment can be produced either by total synthesis (47) or tryptic digestation of the natural peptide (48). The fragments obtained after cleavage with trypsin are separated by reversed-phase HPLC on an Altex Ultrasphere IP column. The isolated N-terminal tryptic fragment (1-14) is conjugated to keyhole limpet hemocyanine for the development of an enzyme-linked immunosorbent assay (ELISA) discriminating between $T\beta_9$ and other structurally closely related ß-thymosins. The specificity of the $T\beta_9$(1-14) antiserum is investigated in a competitive assay, established for $T\beta_9$. The ELISA is found to be linear from 2 to 200 pmol/assay with a lower detection limit of about 2 pmol (Fig.4). The same curve is observed, if $T\beta_9$ is replaced by equimolar amounts of the $T\beta_9$(1-14) fragment. This indicates that the affinity to the antibodies to the fragment (1-14) is comparable to the affinity against the native $T\beta_9$. On the other hand, if antibodies, raised against undigested $T\beta_9$ are used, lower sensitivity is observed. Antisera, raised against $T\beta_9$(1-14) as well as against $T\beta_9$ are used in a $T\beta_9$ assay to investigate the cross-reactivity of the antibodies. Antiserum against $T\beta_9$ cross-reacts 30-40% with $T\beta_4$ while $T\beta_9$(1-14) antiserum cross-reacts less than 1% (Figs 4,5). This indicates that the anti-$T\beta_9$(1-14) antiserum is suitable for studying the levels of $T\beta_9$ in biological extracts. It is obvious that the theoretical considerations are in excellent agreement with the experimental results: The anti-$T\beta_9$(1-14) antiserum shows practically no cross-reactivity with even the structurally closely related $T\beta_4$. Following a corresponding strategy for $T\beta_4$, we also succeeded in the meantime to obtain specific antibodies against $T\beta_4$ (49).

Ubiquitin

According to the Chou-Fasman plot of ubiquitin with marked regions of high antigenic indices (Fig. 6), the conclusion could be drawn that a whole series of peptide fragments should show affinity to polyclonal antibodies produced against the peptide. To proof this suggestion, tryptic fragments are isolated after enzymatic cleavage by means of high performance liquid chromatography (HPLC) (Fig. 7). It is worthwhile mentioning that fragmentation of the peptide could be performed only after destroying the secondary structure of ubiquitin by the addition of a 6 M urea solution. The primary structures of the collected fragments are listed in Table 1. Suprisingly however, none of the isolated peptides showed any affinity to the polyclonal antibodies produced against ubiquitin, though a high affinity is observed against the intact peptide. However, if ubiquitin is incubated with a trypsin solution lacking urea, after 15 min the peptide concentration decreases to zero, as determined with a radioimmunoassay based on our polyclonal antibodies against ubiquitin. By means of amino acid analysis it could be demonstrated that trypsin in urea-free solution cleaves only the C-terminal Gly-Gly residue from ubiquitin and the formed [des-Gly^{75}-Gly^{76}]-ubiquitin is not recognized by our polyclonal antibodies indicating that an interrupted epitope is responsible for the antigen-antibody interaction. These experiments show that polyclonal antibodies, raised against ubiquitin, do not show any affinity to numerous peptides with the natural sequence of the polypeptide, though these peptides are associated with a high antigenic index (50). This example demonstrates that prediction of antigenic determinants may give valuable hints but also fail, especially in the case of interrupted epitopes.

Transmembrane glycoprotein gp41 env fragment

Based on theoretical predictions, the fragment 583-623 of the transmembrane glycoprotein gp41 env should have two major antigenic determinants located in the segment 603-609 and at the C-terminus of the molecule. Therefore, the peptide with the sequence ARILAVERYLKDQQLLGIWGCSGKLICTTAV-PWNASWSNKS, corresponding to the immunodominant region 583-623 on the env reading frame of the HIV-1 isolate BRU and involving the two predicted antigenic sites, is synthesized by the solid phase method. After removal of the protecting groups and cleavage from the Wang resin, the peptide mixture is precipitated with diethyl ether, collected by centrifugation and lyophilized from water.

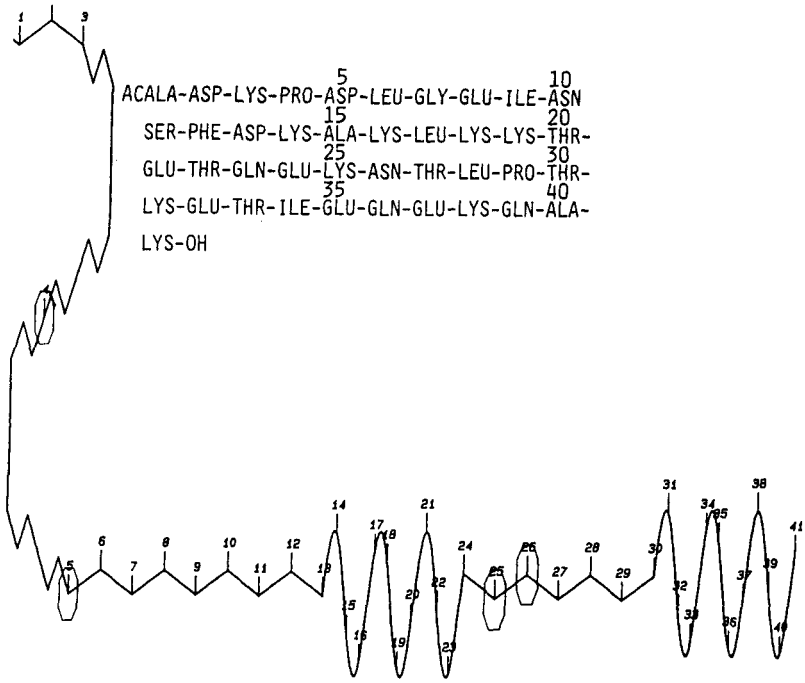

Fig. 3 Chou-Fasman plot of TB$_9$ with marked regions of high antigenic indices. The numbers refer to the TB$_9$ sequence.

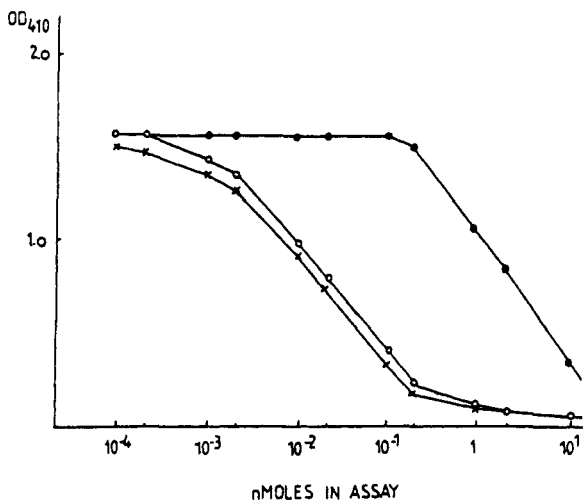

Fig. 4 Displacement curves of TB$_9$ (o), TB$_9$ (1-14) (x) and TB$_4$ (•) in a competitive assay (ELISA) with TB$_9$ bound to microtiter plates using anti-TB$_9$ (1-14) antibodies.

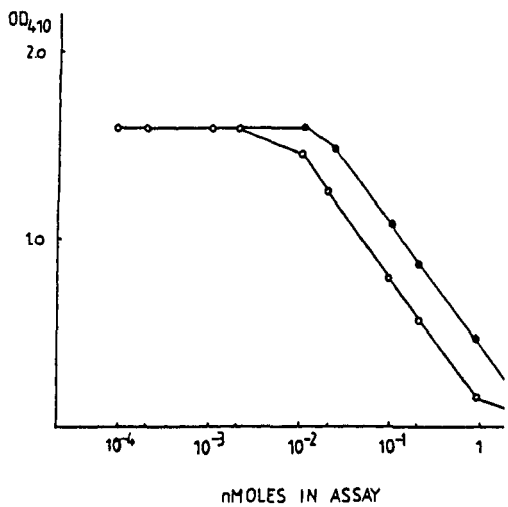

Fig. 5 Displacement curves of TB$_9$ (o) and TB$_4$ (•) in a competitive assay (ELISA) with TB$_9$ bound to microtiter plates. Antibodies uesd in this assay were raised against the undigested TB$_9$.

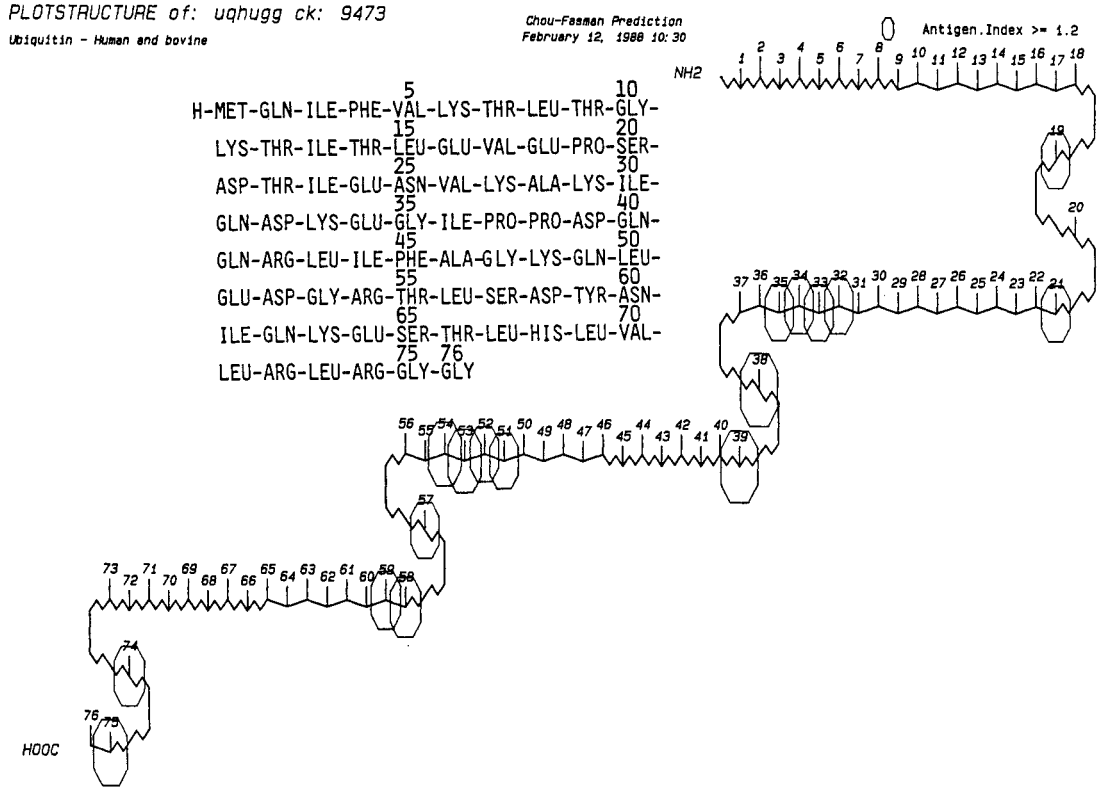

Fig. 6 Chou-Fasman plot of ubiquitin with marked regions of high antigenic indices.

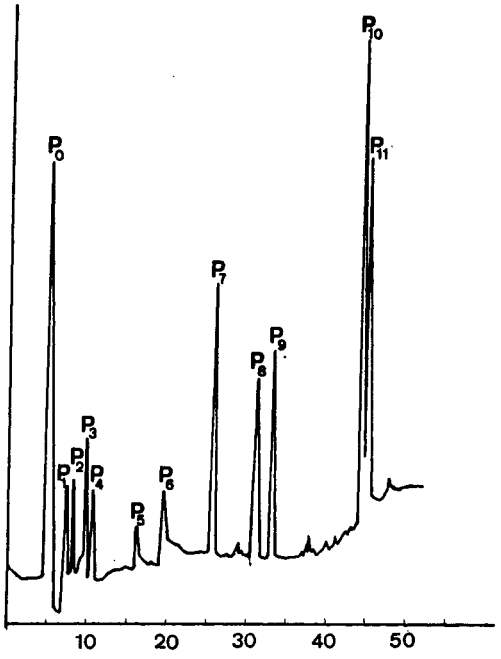

Fig. 7 HPLC of tryptic fragments of bovine ubiquitin. Chromatographic conditions: Column: Nucleosil RP-C_{18} (5μm), 250 x 4 mm; solvent: Linear gradient from 10% B (0,1% trifluoroacetic acid/0,1% triethylamine in 40% acetonitrile) to 70% B in 50 min (A: 0,1% trifluoroacetic acid/0,1% triethylamine in water); flow rate: 1ml/min; detection: UV, λ=206 nm (abs. versus time[min]).

Table 1. Identification of HPLC-isolated tryptic fragments of bovine ubiquitin (chromatographic conditions and numbering see Fig. 7) used for the investigation of their antigen-antibody affinity. Polyclonal antibodies are raised against bovine ubiquitin. The numbers in parentheses refer to the ubiquitin sequence.

Pep. fragm.	Ret. time (min)	Sequence
P_0	5.0	H-Ala-Lys-OH (28-29)
P_1	7.3	H-Ile-Gln-Asp-Lys-OH (30-33)
P_2	8.0	H-Leu-Arg-Gly-Gly-OH (73-76)
P_3	9.3	H-Thr-Leu-Thr-Gly-Lys-OH (7-11)
P_4	10.8	H-Gln-Leu-Glu-Asp-Gly-Arg-OH (49-54)
P_5	16.0	H-Glu-Gly-Ile-Pro-Pro-Asp-Gln-Gln-Arg-OH (34-42)
P_6	19.5	H-Ile-Gln-Asp-Lys-Glu-Gly-Ile-Pro-Pro-Asp-Gln-Gln-Arg-OH (30-41)
P_7	26.0	H-Thr-Leu-Ser-Asp-Tyr-Asn-Ile-Gln-Lys-OH (55-63)
P_8	31.0	H-Leu-Ile-Phe-Ala-Gly-Lys-OH (43-48)
P_9	33.0	H-Met-Gln-Ile-Phe-Val-Lys-OH (1-6)
P_{10}	44.0	H-Thr-Ile-Thr-Leu-Glu-Val-Glu-Pro-Ser-Asp-Thr-Ile-Glu-Asn-Val-Lys-OH (12-27)
P_{11}	45.0	H-Glu-Ser-Thr-Leu-His-Leu-Val-Leu-Arg-OH (64-72)

Two main peptide fractions with molecular masses of 4681 D(I) and 4452 D(II) are obtained, if the crude synthetic product is separated by reversed phase high performance liquid chromatography. Sequence analysis and mass determinations of fractions I and II and their Asp-N fragments showed that fraction I contains the desired peptide sequence, while in the fraction II Lys^{40} is deleted. ELISA measurements, employing these peptides as an antigen adsorbent, have shown to reproducibly detect antibodies in sera of patients with HIV-1 infection. They are also efficiently used in a combined ELISA system for simultaneous detection of anti-HIV-1/2 antibodies and HBsAg. Furthermore, these assays with peptide fractions I and II show significant higher sensitivity for all sera in comparison with the activity of the commercially used 26 amino acid peptide 593-518.

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