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Immunostimulating properties and three-dimensional structure of two tripeptides from human and cow caseins

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Some tripeptides obtained by enzymic digestion of caseins possess immunomodulating properties. In order to correlate activity and structure, X-ray analysis has been applied to two of them Leu-Leu-Tyr and Gly-Leu-Phe.

Immunostimulation; Casein; Immunomodulating tripeptide; 3-dimensional structure

1. INTRODUCTION

The major part of the studies so far devoted to chemically defined immunomodulating substances has been performed with compounds of bacterial origin or with their synthetic analogues [1]. However, we previously observed that enzymic fragments obtained from human casein, the main protein fraction of maternal milk which is usually man's first food, exhibit immunostimulating properties [2]. The purification, the sequence, the synthesis and some biological properties of an immunostimulating hexapeptide (Val-Glu-Pro-Ile-Pro-Tyr) from human β-casein have been described [3]. The presence of some further biologically active short peptides has been detected in human as well as in cow milk caseins. This paper deals with the characterization of two active tripeptides (Gly-Leu-Phe and Leu-Leu-Tyr) and their molecular conformation in the solid state by single crystal X-ray diffraction, as a first attempt to correlate biological activity and structure.

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2. MATERIALS AND METHODS

2.1. *Purification procedures*

Two peptides Gly-Leu-Phe and Leu-Leu-Tyr were obtained from delipidated casein digested by non-pretreated trypsin (Worthington): the digest was filtered on Sephadex G-50 (Pharmacia) [2]. Biologically active fractions were submitted to successive chromatographies. Gly-Leu-Phe, from human casein, was obtained after DEAE-Sephadex A-25 chromatography (fraction III), filtration on Sephadex G-15, Dowex 50×4 chromatography (Bio-Rad) and HPLC [3]. The last HPLC (Waters chromatograph, model ALC/GPC 204) was achieved on a μBondapak C18 column (300×9 mm; Waters associates) eluted at a flow rate of 1 ml/min with a buffer containing 5.5% acetonitrile (Baker chemicals) in 0.1% trifluoroacetic acid (TFA; Uvasol, Merck). Leu-Leu-Tyr, from bovine casein, was obtained after CM-Trisacryl M (IBF) chromatography with a 0.01 M Tris-HCl, pH 4.5, buffer and HPLC (Gilson Chromatograph) with the same column as above. The ultimate HPLC was achieved with a buffer containing 12.5% acetonitrile in 0.1% TFA. The corresponding synthetic peptides were obtained from Bachem.

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2.2. *Biological assays*

2.2.1. In vitro test

Stimulation of phagocytosis of sheep red blood cells (SRBC) by murine peritoneal macrophages [3] was expressed as the increase in the number of macrophages which had at least ingested two SRBC for a total of 100 cells examined. Stimulation of secretion of hemolytic antibodies against SRBC by murine spleen cells [3] was expressed as the increase in the percentage of hemolysis measured by absorbance at 542 nm.

2.2.2. In vivo test

Resistance of mice against infection with *Klebsiella pneumoniae* [3] was tested after administration of various doses of peptides 24 h before infectious challenge. Animals were observed 10 days following infection, mortality being recorded daily.

2.3. *Crystallization*

Leu-Leu-Tyr crystals were obtained from a dimethylformamide/water (2:1, v/v) mixture and Gly-Leu-Phe crystals from a methanol/water (1:3, v/v) one, by slow evaporation at room temperature. Both were very thin needle-shaped crystals elongated along the *c* axis. Leu-Leu-Tyr crystallized as a chloride and Gly-Leu-Phe as a hydrate.

2.4. *X-ray analysis and structure determination*

The crystals were analyzed by X-ray techniques. Parameters and space groups were determined from Weissenberg camera photographs. The data sets were collected on a Philips PW 1100 single crystal automatic diffractometer with CuKα radiation, $\lambda = 0.15418$ nm. The crystals were mounted along the *c* axis. The structure determinations were solved by direct methods [5] and refined by the full-matrix least-squares methods using the Affine program [6] with an isotropic temperature factor for all atoms except the hydrogens.

Table 1

Immunostimulant activities of two casein tripeptides

Peptides	Stimulation of phagocytosis[a]			Enhancement of resistance against *Klebsiella pneumoniae*[b]						
	Concentration (μM)	% stimulation		Way of injection	Doses (mg/kg)	Mean survival time (days)		Survival on day +10	Index of stimulation	$P \leqslant$
		Assay 1	Assay 2			ts	(tc)			
Gly-Leu-Phe	30	146 (28.6)	151 (28)	i.v.	5	4.7	(2.67)	6/15 (4/30)	176	13.55
					1	4.6		4/15	172	1.35
	3	160	157							
				s.c.	5	3.4	(2.67)	3/15 (4/30)	127	41.27
	0.3	127	121		1	5.5		7/15	206	4.19
Leu-Leu-Tyr	30	148 (28.6)	141 (28)	Stimulation of antibody secretion against SRBC[c]						
	3	145	148	Concentration (μM)		% hemolysis		% stimulation	$P \leqslant$	
	0.3	122	128	10		52.6 (45)		116	0.043	
				1		41.0		90.6	0.161	

[a] Figures indicate % increase in phagocytosis over controls $\times 100$. Between parentheses are the values of % phagocytosis in control cultures. Underlined figures are significantly different from controls (*t*-test of Student-Fisher)

[b] An index, ts/tc $\times 100$, was calculated for each experimental group (ts = mean survival time of treated mice, tc = mean survival time of control mice). Statistical analysis was performed by the distribution-free K-sample test of Jonkheere [4] to determine significant results ($P \leqslant 5$, underlined figures)

[c] Figures indicate the % increase in hemolytic antibody secretion over controls $\times 100$. Between parentheses are the values of % hemolysis in controls. Underlined figures are significantly different from controls (*t*-test of Student-Fisher)

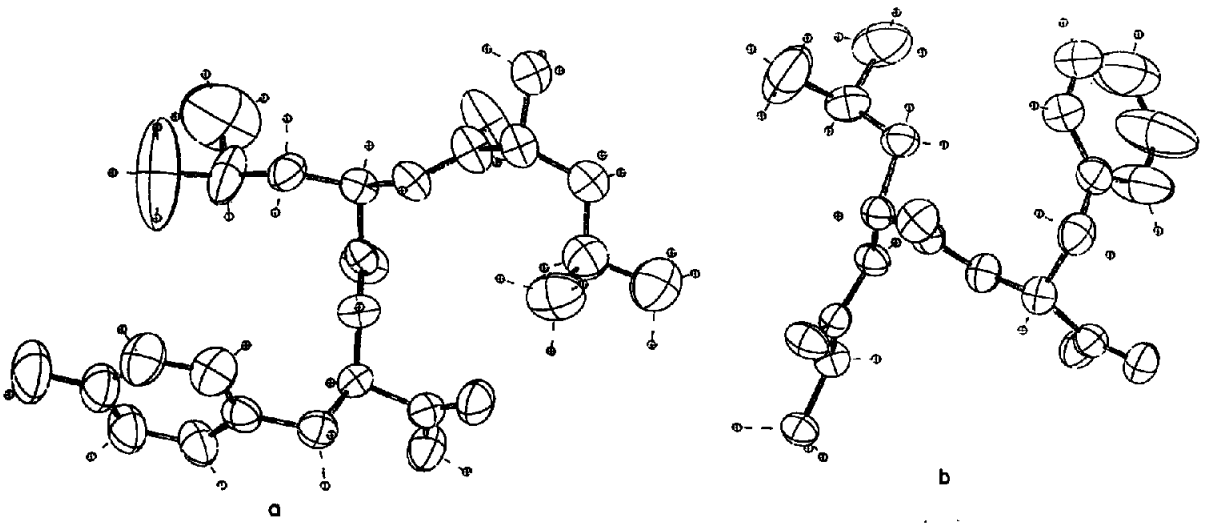

Fig.1. Conformation of Leu-Leu-Tyr (a) and Gly-Leu-Phe (b) tripeptides. Molecular conformations are seen along the *c* axis.

3. RESULTS AND DISCUSSION

3.1. *Immunostimulating properties*

An enzymic digest of human casein was submitted to a series of chromatographic purification steps and of immunomodulating tests, and an active tripeptide was characterized (table 1). The natural as well as the synthetic peptide Gly-Leu-Phe showed a significant activity on the phagocytosis of SRBC by mouse peritoneal macrophages; furthermore it protected the mice against an infection with *Kl. pneumoniae* at 1 mg/kg when administered subcutaneously and to a lesser but still significant degree intravenously. The analogous peptide Gly-Phe-Leu which also occurs in human casein displayed also weak but significant activities when the same biological tests were used. The peptide Leu-Leu-Tyr from cow casein increased again the phagocytosis of SRBC by mouse macrophages but failed to protect the mice against infection with *Kl. pneumoniae;* however it slightly but significantly stimulated the antibody secretion against SRBC by murine spleen cells.

3.2. *X-ray analysis and molecular conformation*

The crystal parameters determined by X-ray techniques were for peptide Leu-Leu-Tyr, which crystallized in the orthorhombic system, space group $P2_12_12_1$: a = 26.074 Å, b = 17.591 Å, c = 5.224 Å. For peptide Gly-Leu-Phe, which crystallized in the monoclinic system, space group B2, the parameters were: a = 18.229 Å, b = 18.781 Å, c = 5.917 Å, γ = 110°65. The two structures were refined up to a R = 8% and 5%, respectively [6].

Fig.1 shows the structure of each peptide molecule. Their molecular conformations are defined by the torsion angles [7] from which some remarks can be drawn: (a) all the peptide bonds are in *trans*-conformation and all Cα-CONH-Cα groups deviate very slightly from planarity (ω: 180°); (b) in peptide Leu-Leu-Tyr, the two ϕ values are of the same magnitude (−100° and −88°) indicating that it is already taking a regular helix conformation, whereas in peptide Gly-Leu-Phe the molecule has a more stretched conformation as the phenylalanine residue has a ϕ value of −174°8; (c) the side chains are very agitated as shown by thermal ellipsoids (fig.1).

The crystal packing of the two tripeptides is shown in fig.2. As expected no intramolecular hydrogen bonds were observed. The molecules are held together through an intermolecular hydrogen bond network. In both peptides hydrogen bonds occur between a carbonyl group and the amino group of a neighbouring molecule. The stacking of the molecules along the *c* axis is a result of the Van der Waals forces. Peptide Gly-Leu-Phe has a zwitterion ion structure as suggested by its carboxyl group dimensions (C-O: 1.22 Å), while the water molecules contribute to the stabilization of the

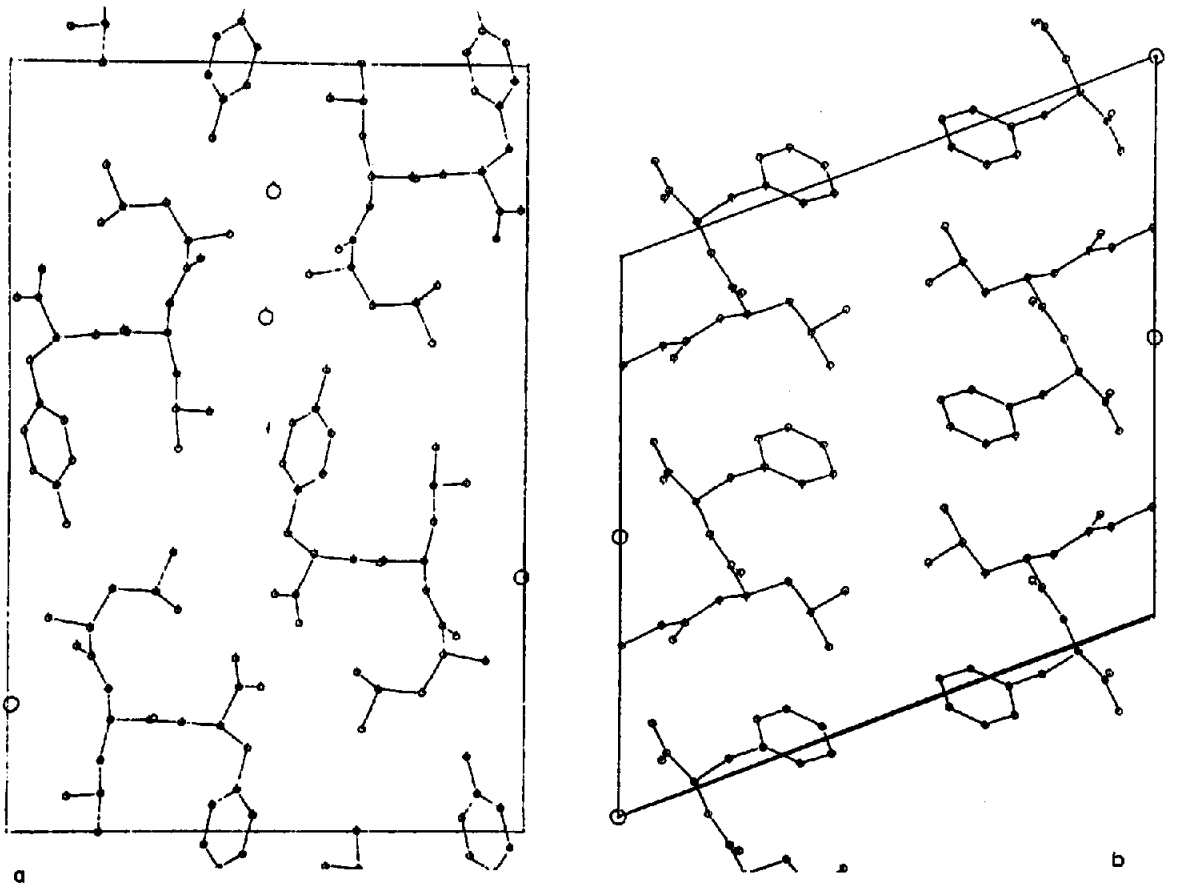

Fig.2. Crystal structures of Leu-Leu-Tyr (a) and Gly-Leu-Phe (b). The crystal structures are viewed along the *c* axis.

whole. In peptide Leu-Leu-Tyr, the amino end is charged (NH_3^+) and stabilized by Cl^-.

The striking feature of the crystal structure of peptide Gly-Leu-Phe is the hydrophobic channel made up of leucine and phenylalanine residues packed together along the *a* axis, while all hydrophilic groups are clustered outside the channel. The segregation of the hydrophobic side chains contributes to stabilize the peptide crystal structure. Whether the extended conformation of this tripeptide is a molecular property or results from crystal packing remains to be checked.

The aim of this study is to initiate a correlation between the structures and biological activities of immunostimulating peptides and later to deduce the topology of their biological targets. The present note reports for the first time the three-dimensional structures of two immunostimulating peptides: other related structures are currently under investigation as obviously further data must be accumulated.

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