Antiviral Compounds Derived from Naturally Occurring Proteins

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Abstract: Food proteins have been considered till recently as substances that provide the organism with essential amino acids necessary for the maintenance of life. In more recent times it has been shown in a convincing manner that they are able to deliver the organism with several bioactive peptides including those possessing antiviral activity. A food protein, lactoferrin, has been shown to inhibit the human immunodeficiency virus, herpes simplex virus, human cytomegalovirus, poliovirus and rotavirus *in vitro* highlighting the importance of naturally occurring food proteins as possible antiviral agents. Of particular interest is that some food proteins, like -lactalbumin or -lactoglobulin, without antiviral activity can be modified, through easy chemical reactions, to acquire antiviral properties. One of the most successful modifications performed on the proteins, consists of chemically modifying the hydrophobic site and the positive charge of lysine and arginine residues of proteins by 3-hydroxyphthalic anhydride. The results reported until now suggest that multiple mechanisms are involved in the inhibition of the viral infection by the proteins investigated. Digestion of some proteins by proteolytic enzymes present in the gastrointestinal tract yields several peptide fragments possessing antiviral properties. Food proteins and their derivatives have the advantage, compared to synthetic pharmacologic compounds, to be non-toxic and surely well accepted by the consumers and thus have great potential for therapeutic application in the future.

INTRODUCTION

Food intake is a process by which organisms are supplied with nutrients necessary for the maintenance of life. Several nutrients are present in the food, for some of them the biological function has been ascertained, whereas for some others this remains only partially known or even unknown. Food proteins have been considered for long time only as compounds, which provide the organisms with amino acids, essential elements for the maintenance of life. Recently it has appeared that this is not their only function, because food proteins are able to deliver to the organism a large number of bioactive peptides, which explicate several biological functions (for an extensive review on the bioactive peptides derived from food proteins see [1]). The spectrum of the biological activity exerted by the peptides derived from food proteins is very wide. It includes antimicrobial, immunomodulatory, antihypertensive, antioxidant and opioid activities. Some of these peptides are already produced on an industrial scale and it is expected that they will find some application, in the near future, as ingredients of "functional food" and pharmaceutical preparation [1].

One class of bioactive peptides, which has attracted in recent time great attention is that of the antimicrobial peptides. Some of them can be delivered into the organism after proteolytical digestion of food proteins. Lactoferrin, for example, a milk protein with moderate bactericidal activity, releases, after its digestion with pepsin, a peptide, the lactoferricin, with high antimicrobial activity [2]. Lysozyme and ovotransferrin from chicken egg white contain in their sequences antimicrobial peptides that can be released by enzymes present in the gastro-intestinal tract [3]. Casein, one of the major component of the milk, releases after its digestion with chymosin two antimicrobial peptide fragments [4].

Recently great attention has been addressed toward food proteins as antiviral agents or as basic substances to develop antiviral compounds. The interest on food proteins as possible substances with antiviral activity derives from the fact that almost all of the antiviral drugs have a limited clinical efficacy, high production costs and toxic side effects [5]. Thus food components would have, compared to chemotherapeutic drugs, the potential advantage to be non-toxic and not expensive to produce. One of the naturally occurring substances, which is mostly investigated for its potential therapeutic use as antiviral compound is lactoferrin. This protein has been shown to be effective against a large number of viruses including HIV and HSV [6-9]. Other food proteins, as for example -lactoglobulin, have been taken as a basis to develop antiviral compounds. This protein does not possess any anti-HIV properties, however its chemical modification by 3-HP yields a compound able to inhibit the multiplication of HIV and HSV[10-13]. In this review we describe several compounds, which possess antiviral activity, derived from food proteins. Their proposed antiviral mechanisms are also discussed. The research on food proteins as antiviral compounds is at the beginning and in future broader and deeper investigations on this area would be desirable.

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CHEMICAL MODIFICATION OF PROTEINS

One of the first systematic attempts to generate compounds with antiviral activity through modification of the amino acid residues of proteins was performed by Neurath *et al.* [10]. During their studies on the design of antiviral compounds directed against HIV-1 [11,12] they found that such compounds, to be effective against this virus, must possess in their structure appropriately spaced and orientated electronegative and hydrophobic sites. It appeared that introduction of both hydrophobic and negatively charged groups into the natural amino acid residues of some proteins will have good chance to provide these latter to acquire binding affinity to viral components and consequently gain antiviral properties. Lysine and arginine residues have been chosen as target residues for the introduction of a negative charge and hydrophobic sites. In a first investigation human and bovine serum albumin as well as casein were chemically modified using several aromatic acid anhydrides (for technical details see Neurath *et al.* [10]). Three derivatives of phthalic anhydride, namely the 3-hydroxyphthalic anhydride, trimellitic anhydride and trimellitic anhydride chloride, were found to be the most effective in converting inactive proteins into compounds with antiviral activity against HIV-1.

The target cells of HIV are CD4+ T lymphocytes and monocytes/macrophages. The protein complex gp120/gp41 on the viral envelope mediates the entry process. In a first step gp120 binds to the CD4 molecule, leading to conformational changes that in turn trigger the exposure of specific sequences on gp120 to the coreceptors CCR5 or CXCR4. Conformational rearrangements of gp41, finally result in the insertion of the fusion peptide into the host cell membrane, ending up with the fusion of the viral and the cellular membranes [13]. Electrostatic interactions seem to play an important role in the contact between the HIV envelope protein and the host cell membrane. Crystallographic data and mutational analysis indicated that the coreceptor-binding site on gp120 forms a positively charged platform that includes the variable V3 loop and is exposed toward the target cell membrane [13]. These processes have been shown to be influenced by negatively charged antiviral substances, which interact with the CD4 receptor [14]. Neurath *et al.* [10] tested extensively the competition of 3-HP-modified compounds using monoclonal antibody-binding assays. From the results obtained they concluded that the competition effect was due to inhibition of the gp120 – CD4 binding process. It was speculated that 3-HP may open the tertiary structure of the modified protein, thus exposing the negatively charged polypeptide sequences [14]. The interaction, however, was not simply related to V3 loop binding, since some modified proteins with antiviral activity did not compete with a V3 loop specific antibody. Furthermore, it was observed that the antiviral effect was not correlated with the extent of modified lysine residues, and consequently not with the net negative charge. Although different mechanisms seem to be involved, the experiments performed by Neurath *et al.* [10] demonstrated that the inhibition of binding between gp120 and the CD4 receptor plays a major role. Based on antibody-binding and –competition tests it was shown that the 3-HP-compounds preferentially bound to gp120-binding sites on the CD4 molecule blocking in this way the virus attachment.

It has been shown that not all proteins gain antiviral properties due to 3-HP modification, indicating that other factors, such as conformational alterations, must be involved in addition [14].

Introduction of negatively charged and hydrophobic sites by succinic anhydride or cis-aconitic anhydride into the protein structure seems to be also a suitable procedure to provide that a protein will gain antiviral properties [15,16]. Both chemical compounds act on the lysine residues of the proteins. Chemically modified proteins with these compounds acquired antiviral activity against both HIV-1 and HIV-2 [15] and against influenza virus [16].

Polyanionic compounds including dextran sulfate and heparin have proven to be able to inhibit HIV-1 and HIV-2 replication [17-20]. However, these substances are far less effective to inhibit HIV-1 in comparison to the proteins modified by aromatic acid anhydrid [10]. Moreover several polyanions present as a drawback to be toxic at chronic administration and interfere with blood coagulation [15].

-LACTALBUMIN

Milk, the first nutrient with which we come in contact in our life, is composed of a number of proteins exhibiting several physiological functions like the delivery of the basic elements to the organism to produce new proteins or to protect it from infectious agents.

Lactose, a nutrient compound present in milk requires for its synthesis, in the lactating mammary gland, an enzymatic system, the lactose synthase. This enzymatic system is composed of two components one possessing catalytic activity and the other having a regulatory function. The catalytic component is represented by the galactosyl-transferase whereas the regulatory component corresponds to -lactalbumin. The function of the galactosyltransferase is to catalyze the transfer of galactose from UDP-galactose into (1 4) linkage with N-acetylglucosamine. Alpha-lactalbumin has the property of modifying the substrate specificity of the galactosyltransferase from N-acetylgluco-samine to glucose allowing the synthesis of lactose [21,22]. Alpha-lactalbumin is a low molecular weight acidic protein (14.2 KDa) present in the whey fraction of milk. Although its function in the maternal organism is well established, its function in neonates is hitherto mostly unknown. Only recently the property to defend the new-born from pathogenic micro-organisms has been putatively ascribed to -lactalbumin [23-25]. Alpha-lactalbumin itself does not possess any antimicrobial activity, however when it is in a particular conformational state it gets antimicrobial properties [23]. In fact, Hakansson *et al.* [23] found that an -lactalbumin folding variant isolated from the human milk showed bactericidal activity against *Streptococcus pneumoniae*. The bactericidal -lactalbumin variant was purified from the casein fraction of milk by ion exchange chromatography. This purification step was very important because it was shown that the native inactive lactalbumin could be converted into the active form by ion exchange chromatography in the presence of a cofactor present in human milk casein [24].

More recently Pellegrini *et al.* [25] have reported that the digestion of -lactalbumin by proteases present in the

gastrointestinal tract yields three bactericidal peptides, which are active essentially against Gram-positive bacteria. Two of those peptides are composed of two polypeptide chains stabilized by a disulfide bridge. The presence of the disulfide bridge is a prerequisite for bactericidal activity.

Another important property ascribed to -lactalbumin is its capacity to bind a number of different metal cations. Alpha-lactalbumin binds with a high affinity to Ca^{2+} and to several other ions like Zn^{2+}, Mn^{2+}, Mg^{2+}, Na^{+}, K^{+} [21]. The binding of such ions, except Zn^{2+}, strongly increases the stability of -lactalbumin. Besides ion binding properties -lactalbumin is able to interact with hydrophobic peptides like mellitin and with lipid membranes [26].

ANTIVIRAL ACTIVITY OF THE CHEMICALLY MODIFIED -LACTALBUMIN

Although the antiviral activity of the native unmodified -lactalbumin has been investigated against several viruses no antiviral property could be ascribed to this protein [27-30]. However, the chemically modified -lactalbumin was shown to possess antiviral activity against HIV [10] and HSV-1 [31]. On the other hand the same chemically modified -lactalbumin failed to inhibit PIV-3 and PRCV [31]. Among all the chemically modified forms of -lactalbumin tested for antiviral activity the 3-HP- -lactalbumin seems to be the most effective. This compound was, among all the other proteinic compounds tested, one of the most potent inhibitor of HIV-1 replication. It had an EC_{50} of 0.1 µM and showed only a weak cytotoxicity with a value of CC_{50} of 50 µM [10]. These values are in the same range as the antiviral activity of AZT, one of the compounds used for a long time in the therapy against HIV-1. The antiviral activity of 3-HP- -lactalbumin was also investigated against HSV-1. Extensive investigations on the antiviral activity of 3-HP- -lactalbumin were performed by Oeverman *et al.* [31]. Those studies comprised either the inhibition of the cytopathic effect induced by HSV-1 or the reduction of the virus yield. The inhibition of the viral infection has been evaluated at various experimental conditions incubating the modified -lactalbumin together with the virus before or during the infection process, or treating the Vero cells with 3-HP- -lactalbumin after virus infection. 3-HP- -lactalbumin was only weakly active when incubated with the cell monolayer before infection, whereas its inhibitory activity against HSV-1 increased considerably when the compound was present during or after infection.

The CC_{50} value was determined to be higher than 8 mg/ml indicating that this compound is poorly or no at all toxic. The EC_{50} ranged between 65µg/ml (compound present during the infection) and 160 µg/ml (compound present after infection).

Herpesviruses enter the cells upon interaction of several envelope glycoproteins with their cell receptors [32]. First, glycoproteins gC or gB bind reversibly to heparan sulfate moieties on the cell surface. Then specific and irreversible interactions with herpesvirus entry receptors follow. In this phase, the interaction between gD and several possible herpesvirus entry mediators, e.g. nectin-1 and nectin-2, plays the major role. However, glycoproteins gB, gH and gL are also essential to induce the fusion process. The antiviral activity of -lactalbumin is thought to be most probably related with receptor competition, mainly by binding to heparan sulfate molecules [31].

A derivative compound of -lactalbumin, namely Suc- -lactalbumin has been investigated for antiviral activity against Influenza virus A and Semliki forest virus. Suc- -lactalbumin was inactive against these two viruses [16].

-LACTOGLOBULIN

-Lactoglobulin is the major whey protein present in the bovine milk. It is present in the milk of ruminants and monogastric animals like horses and pigs but it is absent from the milk of humans and rodents [33,34]. It is an anionic globular protein (pI 4.8) with a molecular weight of about 1.8 KDa. Ruminant -lactoglobulins occur in a dimeric form at a pH range 5 to 8. At the normal pH of milk, which is about 6.5, -lactoglobulin is in a thermodynamic equilibrium between the monomeric and dimeric form [33]. Six genetic variants of the bovine -lactoglobulin, named A, B, C, D, H and W, have been reported until now [34]. Characteristic for -lactoglobulin is its ability to bind ligands of a diverse nature like palmitic acid, cholesterol and retinoic acid [35].

-Lactoglobulin shares strong homology with the human retinol binding protein (25-30 % homology), the main protein involved in the transport of retinol in serum [34,36], however the affinity of -lactoglobulin to retinol is low [34]. Because of its capacity to bind retinol -lactoglobulin is considered as a part of the lipocalin family. Lipocalins are a large and heterogeneous group of extracellular proteins which possess the property to bind hydrophobic molecules, like retinol, and to form stable complexes with other molecules [37]. The group of lipocalins includes invertebrate colorant, pheromone, transport proteins, $_1$-acid glyco-protein, $_1$-microglobulin and prostaglandin D synthase [38].

Some biological functions have been proposed for -lactoglobulin but they have not been substantiated by a definitive experimental evidence till now. It has been speculated that -lactoglobulin could participate in several biological processes like for example in the transport of retinol in the intestine of newborns [39,40], or as a compound that supports the intestinal uptake of retinol [41], or as a protein that binds fatty acids [42].

Despite a large number of intensive investigations concerning physico-chemical, structural and biochemical properties of -lactoglobulin its biological function remains unknown.

ANTIVIRAL ACTIVITY OF -LACTOGLOBULIN

The antiviral activity of -lactoglobulin has been investigated against several viruses like HSV-2 [43], HIV 1 and 2 [27], adenovirus [28], poliovirus [29], rotavirus [30] and hepatitis C virus [44]. Antiviral activity could be ascertained against rotavirus only [30].

ANTIVIRAL ACTIVITY OF THE CHEMICALLY MODIFIED -LACTOGLOBULIN

Several kinds of chemical modifications have been proposed to transform -lactoglobulin in a compound with antiviral properties.

Chemical modification of -lactoglobulin by 3-HP yielded a compound, which exerted antiviral activity *in vitro* against several viruses like HIV-1 and HIV-2 [14,45,46], SIV [45], HSV-1 and HSV-2 [31,47]. Other antiviral compounds have been derived from -lactoglobulin through site specific chemical modification of the amino acid lysin by Suc and Aco [15]. These modified -lactoglobulins exerted antiviral activity against HIV-1 and HIV-2 [15]. The genetic variants A and B of -lactoglobulin have been modified by 3-HP and investigated for their antiviral activity by Neurath [46] against HIV-1. The inhibitory activity of the 3-HP variant of -lactoglobulin was determined by measuring the inhibition of the binding between HIV-1 envelope glycoprotein gp120 and soluble CD4. The EC_{50} values for inhibition of gp120-CD4 binding were found to be 11.2 nM for the modified variant A and 2.6 nM for the variant B, respectively. In comparison the EC_{50} for the inhibition of the nucleocapsid protein p24 production ranged between 55.6 nM (variant A) and 39.4 (variant B) [46]. Swart *et al.* [27] investigated the binding capacity of the Suc- and Aco- modified variants of -lactoglobulin A and B, respectively, toward loop V2 and V3 sequences of the HIV-1 envelope glycoprotein gp120. From the results obtained by their experiments they found EC_{50} values in a range between 500-3000nM for the Suc- or Aco- -lactoglobulin A and B [27]. Since sexual contact is the most frequent route of HIV transmission, Neurath *et al.* [48] investigated the antiviral activity of 3-HP- -lactoglobulin against HIV-1 in conditions similar to that present in the acidic vaginal environment in presence of seminal plasma. It was found that the inhibitory activity of 3-HP- -lactoglobulin is affected by the pH and by components present in human seminal plasma. In particular it was found that the binding capacity of 3-HP- -lactoglobulin to both soluble and insoluble CD4 is, in an acidic environment, decreased in comparison with the binding observed at neutral pH. The seminal plasma contains Zn++ ions which bind to 3-HP- -lactoglobulin, thus decreasing the binding capacity of 3-HP- -lactoglobulin to CD4. The negative effects due to the acidic pH and the presence of Zn++ ions, can be compensated in two ways, either by increasing the concentration of 3-HP- -lactoglobulin or by addition of the chelating agent EDTA to the medium [48]. Pretreatment of HIV with 3-HP- -lactoglobulin also decreased infectivity, indicating a virucidal effect of the modified protein. However, the virucidal effect of 3-HP- -lactoglobulin was detected only at a higher concentration [49].

In another investigation it was shown that 3-HP- -lactoglobulin is able to inhibit the uptake of HIV-1 by CD4- epithelial cell lines, i.e. human adenocarcinoma and cervical carcinoma cells [50]. Indeed, the virus uptake was blocked by 3-HP- -lactoglobulin as efficiently as in CD4+ cells. It was additionally shown that the gp120 V3 loop was involved in the virus-cell binding. Galactosyl ceramide has been postulated by some authors to act as an additional coreceptor for HIV but this was not detectable on the epithelial cells used [50].

These results suggest that 3-HP- -lactoglobulin has a potential for prevention of sexual transmission of HIV-1 through epithelial cells. The binding kinetics between 3-HP- -lactoglobulin and the CD4 receptor was investigated by using the surface plasmon resonance technology, which allows quantitative analysis of molecular interaction in real time [30]. The association and dissociation constants were evaluated to be ka=2.4 x 10^6 $M^{-1}s^{-1}$ and Kd= $2.3x10^{-4}s^{-1}$, respectively, indicating a high affinity between the modified -lactoglobulin and the CD4 receptor. The complex 3-HP- -lactoglobulin -CD4 receptor is very stable as shown by its $t_{1/2}$ value of 2 h.

Recently, in order to understand the possible action mechanism of the modified -lactoglobulin molecules against HIV the cationic forms of -lactoglobulin A and B were chemically prepared and investigated against HIV-1, HIV-2 and HCMV [51,52]. The addition of positive charges to the -lactoglobulin molecules resulted in a moderate anti-HIV-1 activity. On the contrary high anti-HCMV activity was detected for the positively charged -lactoglobulin [52]. 3-HP- -lactoglobulin inhibited the transmission of SIV *in vivo* quite efficiently in a rhesus monkey model. It has been shown that 3-HP- -lactoglobulin suspended in PBS protected completely three out of six monkeys against vaginal transmission of SIV [53]. Two of the other three treated infected monkeys presented, however, symptoms of a SIV infection retarded of 4 and 12 weeks compared to the control animals [53].

3-HP- -lactoglobulin inhibits the infection of HSV-1, HSV-2 and HCMV [31,47]. HSV-1 seemed to be the most sensitive to the action of 3-HP- -lactoglobulin compared to HSV-2 and HCMV [47]. Of special interest is the inhibition of HSV cell-to-cell spread, which was demonstrated by mixing infected cells, expressing the lacZ gene with uninfected cells without the marker. Studies using monoclonal antibodies demonstrated that binding of anti-gE and anti-gC antibodies to their corresponding virus epitopes was influenced by 3-HP- -lactoglobulin. These results confirm the importance of the interaction of 3-HP- -lactoglobulin with the cellular heparan sulfate proteoglycan receptor for herpesvirus glycoprotein gC, and in addition explain the observed inhibition of cell-to-cell spread, for which gE plays a major role.

Pretreatment of HSV-1 and HSV-2 with 3-HP- -lactoglobulin also decreased infectivity, indicating a virucidal effect of the modified protein, as it was also found for HIV [49].

The efficiency to inhibit HSV-1 by 3-HP- -lactoglobulin depended of the assay conditions, e.g. whether the 3-HP- -lactoglobulin was present before, during or after virus infection [31]. 3-HP- -lactoglobulin inhibited HSV-1 more efficiently when present during or after virus infection of the cells. Under these conditions the antiherpetic activity of 3-HP- -lactoglobulin revealed an EC_{50} value of 30 µg/ml, whereas the EC_{50} value increased to 465 µg/ml when the monolayer was treated before virus infection [31]. Similar results have been reported by Neurath and co-workers [47]. Kokuba *et al.* [43] tested the influence of 3-HP- -lactoglobulin on the genital HSV-2 infection in a mouse model. The simultaneous exposition to HSV-2 and 3-HP- -lactoglobulin caused a significant decrease in virus shedding, in the lesion development and in mortality in the infected mice in comparison to the control animals treated with PBS. When formulated in a gel which allowed a prolonged action

of 3-HP- -lactoglobulin in the vagina a strong increase of the antiviral activity was observed when the compound was administrated before virus infection. However the 3-HP- -lactoglobulin was ineffective to protect the animals when it was administrated during or after the HSV-2 infection indicating that 3-HP- -lactoglobulin interferes with the binding of the virus to the target cells [43]. This result is in contrast with the results reported for the antiviral activity of 3-HP- -lactoglobulin against HSV-1 *in vitro* where the maximal efficiency of 3-HP- -lactoglobulin to inhibit the spread of infection was reached when the compound was given after viral infection [31]. Although 3-HP- -lactoglobulin proved to have potent antiviral activity against HSV-1 and HSV-2 it failed to inhibit other enveloped viruses, such as PIV-3 and PRCV [31].

Aco- -lactoglobulin A strongly inhibited the fusion of the influenza virus hemagglutinin with erythrocyte ghosts [16]. Since not all proteins investigated modified in the same way had this inhibitory effect it was concluded that the negative charge density of Aco- -lactoglobulin A had no influence on its antiviral activity. Based on corresponding assay conditions it was also shown that the inhibition of hemagglutinin mediated membrane fusion occurred at some step after binding virus to the cell and acidification of environment. These results led to the conclusion that the fusion step itself was affected.

LYSOZYME

Lysozyme (also known as muramidase) was discovered by Fleming in 1922 [54] to be an antibacterial enzyme capable of hydrolyzing the peptidoglycan layer of the cell wall in some gram-positive bacteria [55]. Since its discovery it has been subjected to extensive physico-chemical and microbiological investigations. Chicken egg white lysozyme was the first protein to be sequenced and the first enzyme subjected to X-ray crystallographic analysis [56]. Lysozyme is widely distributed in nature and found in many cells, tissues and secretions from a multitude of organisms [57,58]. Besides its antimicrobial activity, lysozyme has many other functions, including anti-inflammatory activity [59], enhancing phagocytic activity of polymorphonuclear leukocytes [60] and macrophages [61], stimulation of monocytes [62], antitumor activity [63] and induction of fusion of phospholipid vesicles [64].

Lysozyme is a cationic protein of 14.4 KDa, which structurally consists of two domains separated by a deep cleft across its entire width. One domain consists predominantly of helical structures and the other of -sheets [65]. The protein structure is stabilized by four disulfide bridges. The natural substrate of lysozyme is the peptidoglycan (murein) polymer, a molecule present only in the prokaryotic cells. The hydrolysis of the peptidoglycan of the bacterial cell wall leads to bring about the bacterial death.

Until recently it has been believed that lysozyme is ineffective against Gram-negative bacteria because it was considered unable to penetrate the outer membrane, which protects the peptidoglycan layer of the Gram-negative bacteria. However, in recent times, evidence has been presented that the bactericidal spectrum of lysozyme is wider than supposed and includes the Gram-negative bacteria as well [66]. These species are killed by lysozyme following a mechanism which does not necessarily involve its muramidase activity because dead bacteria do not show any sign of lysis [67,68]. Another experimental result that confirms the independence of the bactericidal properties of lysozyme from its enzymatic activity consists in the fact that denatured lysozyme, which has lost its catalytic activity still retains its bactericidal properties [69-71].

Three kinds of lysozyme, named c, calcium-binding c and g, which differ from each other in molecular weight and amino acid sequences, have been found in vertebrates and invertebrates. However, the lysozyme g seems to be present only in the avian egg white [56].

ANTIVIRAL ACTIVITY OF LYSOZYME

Antiviral properties have been ascribed to lysozyme. Anti HIV activity of the chicken and the human lysozyme has been reported by Lee-Huang [72]. The antiviral activity of lysozyme against HIV was supposed to be due to the properties of lysozyme to digest the viral polysaccharides. However, Steinrauf *et al.* [73] provided evidence that lysozyme is able to bind DNA molecules through electrostatic interaction. The authors speculated that lysozyme may have a functional role in the regulation of DNA and RNA that might also be responsible for the antiviral effect by operating on the processes of transcription and replication.

Antiherpetic activity of lysozyme has been recently reported [74]. The EC_{50} value was determined to be 497 μg/ml. It appeared that lysozyme has a synergistic effect on glycyrrhizic acid enhancing the antiherpetic effect of this molecule. Interestingly, the same authors were also able to reveal a synergistic effect between the lactoferrin and lysozyme when these molecules were assayed together against HSV-1. A weak antiherpetic activity of lysozyme has also been reported by Cisani *et al.* [75]. The same authors presented evidence later that lysozyme is able to inhibit the cell fusion induced by HSV blocking in this way the spread of the viral infection [76]. Lysozyme preincubated with HSV-1 did not exert any virucidal effect. The antiherpetic properties of lysozyme resulted independently from its enzymatic activity since the heat denatured lysozyme showed a higher antiherpetic activity than the native molecule [75,76]. Recently, we investigated the antiviral properties of lysozyme against HSV-1, PIV-3 and PRCV [31]. The results showed that lysozyme does not possess any antiviral activity against these viruses. Thus, whether lysozyme possesses any antiherpetic activity must still be definitively demonstrated.

Antiviral properties of lysozyme against influenza and viral hepatitis have been described by Hasselberger [59]. However, neither the experimental results or the assay conditions have been reported.

ANTIVIRAL ACTIVITY OF THE CHEMICALLY MODIFIED LYSOZYME

Lysozyme has been subjected to several chemical modifications with the aim to increase its bactericidal activity [77]. In this context Ibrahim *et al.* [77,78] succeeded by fatty-acylation of the lysine residues of the lysozyme

molecule. In addition, chemical modification of lysozyme by coupling perillaldehyde to the lysine residues of lysozyme yielded several derivatives with higher bactericidal activity than the parent molecule [77]. An increase of the bactericidal activity of lysozyme was also obtained by genetic fusion of hydrophobic peptides of different lengths to the C-terminus of lysozyme [77,79]. Although modified lysozyme acquired new antimicrobial properties, these compounds have never been tested for antiviral activity.

Guanidyl-lysozyme, an enzymatically active derivative of chicken egg white lysozyme, lysozyme methyl ester and guanidyl-lysozyme methyl ester, two enzymatically inactive derivatives of lysozyme, have been investigated for their antiviral activity against HSV [75,76]. These compounds showed a marked inhibition of the cytopathic effect induced by HSV in cell culture.

Lysozyme modified by 3-HP was tested recently against HSV-1, PIV-3 and PRCV. Only HSV-1 was found to be sensitive to 3-HP-lysozyme [31]. The antiviral effect of this compound against HSV-1 was investigated under several assay conditions. 3-HP-lysozyme explicated the most efficient inhibitory activity when administered to the cell monolayer after viral infection. The EC_{50} value determined at this experimental condition was 6 µg/ml. 3-HP-Lysozyme was also able to protect the cells when administered during HSV-1 infection with an EC_{50} value of 14 µg/ml. The capacity to inhibit HSV-1 by 3-HP-lysozyme greatly decreased if the cells were treated before infection with HSV-1. In this case the EC_{50} value was 170 µg/ml. Administered at very high concentration 3-HP-lysozyme had some toxic effects on the cells with a CC_{50} value of 5 mg/ml.

LACTOFERRIN

Lactoferrin is an iron-binding protein, which belongs to the transferrin family. It is a glycoprotein composed of a single polypeptide chain having a molecular weight of 80 KDa. It is mainly present in milk and in lower extent in bile, tears and in the granules of neutrophils. Although lactoferrin shares a similar structure with the other members of the transferrin family it is distinguished from transferrin by the fact that its affinity to iron is 250-fold greater than that of transferrin. Moreover lactoferrin possesses a strongly basic region which is on account of its high pI (pI 9.0) compared with 5.5 for transferrin [80]. Beside iron, lactoferrin can also bind other metal ions like copper, manganese, aluminum [81], however these ions bind to lactoferrin in a lower extent than iron. In fact human lactoferrin was found to contain 2000 times more iron than manganese [82]. Whether the binding of lactoferrin to other ions than iron has a biological meaning remains to be demonstrated. Lactoferrin is expressed in several organs under different control mechanisms. In the granules of neutrophils the expression of lactoferrin is part of the maturation process, whereas in the mammary gland lactoferrin expression is hormonally regulated by prolactin, and in the uterus its production is regulated by 17 -estradiol [83]. There is great variation in the lactoferrin content of the milk of different species. In some species like human, pig and mice the lactoferrin concentration in milk is high. The concentration of lactoferrin in human colostrum can be as high as 80 mg/ml, which then drops to 10 mg/ml during the lactogenesis [84]. In the milk of some species like cattle and other ruminants the lactoferrin concentration is low, and the milk of other animals such as rats, for example, does not contain lactoferrin at all [83]. Many biological functions have been attributed to lactoferrin. These include iron transport, as well as growth factor, immunoregulatory, antiviral, bacteriostatic and bactericidal functions [80,83,85]. In the last time the bacteriostatic and bactericidal properties of lactoferrin have attracted a great interest. One of the first functions ascribed to lactoferrin was the inhibition of the bacterial growth. It was supposed that lactoferrin would hinder the iron utilization by bacteria leading to bacteriostasis. For its bactericidal properties lactoferrin is considered as one of the components of the innate immunity. However, it has been shown later that the bactericidal activity of lactoferrin is independent from its capacity to sequester iron, because iron saturated lactoferrin still possessed antibacterial activity [85]. Bellamy *et al.* [2] were able to isolate the bactericidal domain of the human and the bovine lactoferrin. The bactericidal domain is composed of 18 amino acids (residues 20-37 of the human and 19-36 of the bovine lactoferrin, respectively) and is located in the basic N-terminal region of the protein, which is distinct from the iron-binding site of lactoferrin. The peptides corresponding to the bactericidal domain of lactoferrin have been called lactoferricin H (human) and lactoferricin B (bovine). They possess a higher bactericidal activity and a broader spectrum of action than the parent molecules [2,86].

ANTIVIRAL ACTIVITY OF LACTOFERRIN

Breast milk contains several components with antimicrobial activity. However most of the antiviral activity detected in milk can be ascribed only to lactoferrin [9]. The antiviral activity of lactoferrin is directed against a broad spectrum of viruses, including both RNA- and DNA-viruses, enveloped as well as naked viruses. Lactoferrin saturated with iron ions has been shown to exhibit antiviral activity against HSV-1 and HSV-2 [6,8,87,88] with EC_{50} values of 12 µg/ml and 5.2 µg/ml for HSV-1 and HSV-2, respectively. The bovine lactoferrin was more active than the human lactoferrin. The antiviral activity seemed not to be associated with the presence of metal ions in the lactoferrin molecule because apolactoferrin and metal saturated lactoferrin had a similar antiviral activity. In fact the EC_{50} values of 28 µg/ml and of 31 µg/ml determined for apolactoferrin against HSV-1 and HSV-2, respectively, are very near to those found for the metal saturated form [87]. Lactoferrin was supposed to block herpetic infection at the virus adsorption phase by interaction with heparan sulfate, a proteoglycan present on the cell membrane [8]. Marchetti *et al.* [8] suggested that the N-terminal part of lactoferrin might be responsible for competition with the virus attachment. Administration of lactoferrin after cell infection had no appreciable effect on the virus multiplication [87]. Antiherpetic activity of lactoferrin *in vivo* has been reported by Fujihara and Hayashi [89] in a mouse cornea model. Administration of lactoferrin to the herpes virus infected cornea reduced greatly the severity of damage without, however, eliminating the virus.

Antiviral activity against HCMV, another virus of the herpes virus family, was shown with both bovine and the

human lactoferrin. The EC_{50} values were determined to be 36 µg/ml and 90 µg/ml, respectively [7]. Lactoferrin probably exerts its effects against HCMV at the level of virus adsorption or penetration because once the cells have been infected the protein was no more able to develop any antiviral action. Full protection was achieved when lactoferrin and HCMV were mixed together before being added to the cell monolayer [7]. The protection was supposed to be possibly due to the binding of the lactoferrin molecule to the cell membrane [7]. A direct interaction of the protein with the virus, however, could not be excluded. Almost complete protection has been observed when the cells were preincubated 30 min with lactoferrin before the virus was added [7]. The N-terminal region of lactoferrin seemed to be essential for its antiviral activity [9]. Lactoferrin also had a protective effect *in vivo* as demonstrated in a mouse model. Animals treated with lactoferrin survived an infection with a lethal dose of MCMV. The antiviral effect of the protein was optimal when the treatment was started before infection [9]. Antiviral activity of lactoferrin against HIV virus has also been described. Harmsen *et al.* [7] could show that among several proteins tested against HIV-1, lactoferrin was the only one that inhibited HIV-1 replication in MT4 cells. The inhibition values reported for the bovine and human lactoferrin were 40 µg/ml and 75 µg/ml, respectively. Puddu *et al.* [90] reported that the inhibition of the HIV-1 replication in the C8166 T-cell line by lactoferrin was independent of metal ion saturation. The inhibition values were determined to be 6.5 µg/ml for the bovine apolactoferrin and 2.0 µg/ml for the Fe^{3+} saturated form. The inhibition of the HIV-1 replication was markedly better when the protein was added prior to HIV-1 infection or was present during the virus adsorption step. It has been suggested that lactoferrin is able to bind to the V3 domain of gp120 of HIV [91]. It is interesting to note that the antibacterial domain does not exhibit any antiviral activity against HIV [13].

Lactoferrin may also exhibit antiviral properties against HCV. Ikeda *et al.* [92] have shown that lactoferrin is able to prevent the infection of HCV in the cultured human hepatocyte cell line PH5CH8. Since preincubation of lactoferrin and HCV was required to prevent infection of the cells it was hypothesized that the inhibition happens through a direct interaction between the virus and lactoferrin. This idea is supported by the fact that preincubation of the PH5CH8 cells with lactoferrin had no inhibitory effect on HCV, indicating that the antiviral activity of lactoferrin against HCV was not due to the interaction of lactoferrin with the cells.

It was also shown that heat denatured lactoferrin or its fragment lactoferricin abolished the anti-HCV activity, suggesting that the natural conformation of the protein is a precondition for its activity.

It has been shown later that the domain responsible for the antiviral activity of lactoferrin against HCV does not coincide with its bactericidal domain [44]. Bovine and human lactoferrins are able to bind to the HCV envelope proteins E1 and E2. This binding inhibits any possible interaction of the virus with its putative cellular receptor CD81. Nozaki *et al.* [93] analyzed the HCV glycoprotein E2 recognition site of lactoferrin by peptide-binding assays. They found a C-terminal peptide of 93 amino acids, exhibiting a partial homology to CD81. They could moreover show that the antiviral properties of the 93 peptide were confined to a 33-peptide only.

Furthermore, the same authors provided evidence that lactoferrin is able to achieve antiviral activity against hepatitis G virus *in vivo*. In a recent clinical pilot study lactoferrin was shown to be effective in patients suffering of hepatitis. After eight weeks of treatment with 1.8-3.6 g/day a regression of the clinical symptoms was observed [94].

Antiviral activity of lactoferrin against rotavirus has been reported recently by Superti *et al.* [95,96]. These authors have also investigated the anti-rotaviral activity of lactoferrin saturated with different metal ions. The values of the antiviral activity of the different metal saturated lactoferrins were found to be very close to each other. The EC_{50} values ranged between 47 µg/ml for the iron saturated lactoferrin, and 62 µg/ml for the manganese- and zinc saturated lactoferrin. It has been shown that both the apo and the saturated lactoferrin are able to inhibit viral attachment, mainly by binding to the virus particles. The same authors have also reported and speculated that desialysation of lactoferrin enhanced its anti rotavirus activity supporting the idea that sialic acid present on the cell surface may serve as receptor for rotavirus [96].

Anti-hantaviral activity *in vitro* and *in vivo* of lactoferrin has been reported recently [97,98]. Pretreatment of Vero E6 cells with lactoferrin greatly reduced the multiplication of the hantavirus. The EC_{50} value was determined to be 39 µg/ml. Post-infection treatment of the cells with lactoferrin was only weakly effective. In this case the EC_{50} value was determined to be 2500 µg/ml. Pretreatment of hantavirus infected suckling mice with 160 mg lactoferrin/kg body weight increased the survival rate from 7% (without treatment) to 94% [98].

Both bovine and human lactoferrin were found to possess antiviral activity against enterovirus 71, a virus that is responsible for severe neurological diseases in children [99]. *In vitro*, bovine lactoferrin was more effective than the human lactoferrin, with EC_{50} values of 10.5 µg/ml and 103.3 µg/ml, respectively [99]. It has been supposed that lactoferrin exerts its antiviral activity against enterovirus 71 at the level of viral adsorption because the ongoing infection could not be further inhibited after the virus had penetrated the RD cells [99].

Native lactoferrin and lactoferrin molecules saturated with several different ions have been shown to be able to prevent the poliovirus infection [100]. The EC_{50} values ranged between 370 µg/ml for the human and 650 µg/ml for the bovine lactoferrin.

Anti-adenovirus activity of lactoferrin has recently been reported in addition [101,102]. EC_{50} values determined for the human and bovine lactoferrin were 0.56 µg/ml and 0.08 µg/ml, respectively. It has been shown that the inhibition of adenovirus multiplication takes place during the virus attachment step, mainly through competition for the common glycosaminoglycan receptor on the cell membrane. Di Biase *et al.* [102] studied extensively the mechanism of lactoferrin

against adenovirus type 2. They found that the N-lobe of lactoferrin was the most important domain for anti-adenovirus activity. Their data provided evidence that the anti-adenovirus activity is mediated by the cluster of positive charges at the N-terminus of the lactoferrin molecule. They showed moreover that the peptide lactoferricin alone is sufficient to prevent viral infection. Similar results have been reported by Arnold *et al.* [101].

It is to keep in mind that besides specific interactions with virus replication lactoferrin may also explicate its antiviral action stimulating the immune system. In fact lactoferrin is able to activate natural killer cells, to influence levels of inflammatory mediators, and to inactivate microbes in neutrophils [103,104].

ANTIVIRAL ACTIVITY OF THE CHEMICALLY MODIFIED LACTOFERRIN

The antiviral activity of several chemically modified lactoferrins has been investigated against a number of viruses. Asialo-lactoferrin, i.e. lactoferrin treated with neuraminidase was investigated against HIV-1 and HCMV [7]. Both, the native and the enzymatically modified lactoferrin presented a similar antiviral activity against these two viruses. When investigated against HIV under the same assay conditions the EC_{50} values were 39,6 µg/ml for the native lactoferrin and 46 µg/ml for the asialo-lactoferrin, respectively. Both lactoferrin forms assayed against HCMV presented the same EC_{50} value of 36.7 µg/ml [7]. Acylation of lactoferrin using Aco- or Suc-anhydrides increased the anti-HIV-1 and anti-HCMV activity. The EC_{50} values of Suc- and Aco- lactoferrin for HIV-1 have been determined to be 12.3 and 2.0 nM, respectively [105]. These derivatives of lactoferrin were ineffective against HIV-2, simian immunodeficiency [105] and influenza virus [106].

CASEIN

Caseins are a family of milk proteins, which represent the main protein component of the milk. Caseins are not present in milk as single elements but as large complexed structures containing besides caseins also calcium phosphate [107]. Four caseins, namely $_{s1}$-casein, $_{s2}$-casein, -casein and -casein, differing from each other in their amino acid composition and in their phosphorylation- and glycosylation-sites, have been reported until now. Another one, the -casein, is a product derived from the proteolytical action of plasmin on -casein. Several genetic variants have been described for caseins. There are five known genetic variants of $_{s1}$-casein, four of $_{s2}$-casein, seven of -casein and two of -casein [107]. Caseins are acidic proteins with a pI range between pH 4.9 and 5.9. Their molecular weights vary between 19 KDa for -casein and 25 KDa for $_{s2}$-casein. Although hitherto no biological activity has been ascertained for the caseins they are nevertheless considered as an important nutritional component, which constantly provides bioactive peptides to the organism. In fact a large number of bioactive peptides derived from the proteolytical hydrolysis of caseins have been isolated and characterized. These peptides possess several biological activities like antimicrobial activity [108], opioid agonistic and antagonistic activities [109], immunomodulatory effects [110], mineral binding properties [111], antihypertensive effect [112] and antithrombotic activity [113].

ANTIVIRAL ACTIVITY OF THE CHEMICALLY MODIFIED CASEINS

No antiviral activity has been ascribed to the caseins. However, chemically modified caseins may acquire antiviral properties. Several modifications of the casein molecules have been analyzed by Neurath *et al.* [10]. The modified caseins were successfully tested against HIV-1. Casein modified with phthalic anhydrid had EC_{50} values ranging between 8.5 µg/ml and 12.8 µg/ml. Similar values have been obtained by modification of casein with phenylglyoxal (EC_{50} = 13.6 µg/ml) or with 3-HP (EC_{50} = 11.5 µg/ml). Casein modified with cis-aconitic anhydrid was found to be less efficient against HSV-1 in comparison to the modifications mentioned above. Its EC_{50} value was determined to be 79.8 µg/ml. Berkhout *et al.* [14] have modified s2-casein by 3-HP. They confirmed the results reported by Neurath *et al.* [10], showing that this compound possesses antiviral activity against HIV. With an EC_{50} value of 0.1 µM its antiviral activity was in the same range as that of AZT [14]. Suc- and Aco- casein possessed also antiviral activity against HIV-1. Their EC_{50} values were 2.2 µM and 0.5 µM respectively [105]. The same compounds failed to inhibit the multiplication of HIV-2. In an attempt to get new antiviral compounds the cationization of -casein, using anhydrous ethylene diamine, was performed by Swart *et al.* [105]. The cationized casein failed to inhibit both HIV-1 and HCMV. The inhibition of the binding of HIV gp120 to the CD40 receptor has been suggested as a possible mechanism of action exerted by the Suc- and Aco-modified casein [10,114].

ANTIVIRAL PEPTIDES DERIVED FROM FOOD PROTEINS

Antiviral peptides derived from food and other proteins have only been poorly investigated. Digestion of aprotinin, a protease inhibitor present in several bovine organs and tissues, by clostripain yielded a peptide with antiviral activity against HSV-1 and PIV-3 [115]. The peptide, YFYNAK, with the sequence corresponding to the amino acid residues 12-26 of aprotinin showed an EC_{50} value of 38 µM when tested against HSV-1. The peptide failed to inhibit EHV-1 and PRCV. Its antiviral activity has been investigated under several assay conditions. The peptide was able to inhibit HSV-1 and PIV-3 replication only after infection of the cells. Siciliano *et al.* [116] digested bovine lactoferrin proteolytically by trypsin and tested the resulting tryptic fragments against HSV-1. Two large fragments were isolated and shown to possess antiviral activity against HSV-1. They corresponded to the N-terminal part (amino acid residues 1-280) and to the C-terminus (amino acid residues 345-689) of lactoferrin. The EC_{50} values determined for the peptide 1-280 and for the peptide 345-689 were 25 µg/ml and 320 µg/ml, respectively. These EC_{50} values were higher than that determined in parallel for the native lactoferrin (EC_{50}= 10 µg/ml). Two other peptide fragments of lactoferrin (amino acid residues 222-230 and 264-269) were isolated with apparently no antiviral activity. However an antiviral activity against HSV-1 was observed when both peptides were tested

in a 1:1 stoichiometric association. The EC_{50} value of these two fragments assayed together was determined to be 850 µg/ml [116]. The tryptic fragments of bovine lactoferrin have also been investigated for antiviral activity against rotavirus [117]. Two tryptic fragments of lactoferrin corresponding to the amino acid residues 86-258 and 324-329, respectively, showed antiviral activity against rotavirus, but to a lower extent than the parent protein (EC_{50} = 50 µg/ml). The EC_{50} values determined for these peptides were 125 µg/ml for the fragment 86-258 and 1000 µg/ml for the fragment 324-329 [117]. More recently Berkout *et al.* [13] reported the anti-HIV activity of a synthetic fragment of lactoferrin (amino acid residues 17-41). The antiviral activity of this peptide, however, was weaker than that of the native lactoferrin. Fragments, positively and negatively charged, derived from caseins were investigated for anti-HIV activity. None of the fragments exhibited any antiviral activity [13,114].

A recent study performed in our laboratory provided evidence that a large number of antiviral peptides can be generated from the proteolytical digestion of -lactalbumin , -lactoglobulin and lysozyme [118]. These proteins were digested by proteases present in the gastro-intestinal tract of mammalians, namely trypsin, chymotrypsin and pepsin. The antiviral activity of the peptide fragments against HSV-1 has been examined under different assay conditions, i.e. pretreatment of the cells, and treatment during or after infection of Vero cells. As a first screening the examination of the antiviral activity was restricted to the pools isolated from the reversed phase chromatography of the digested proteins. Although the single peptides were not isolated, the results indicated that peptides with antiviral properties were present in the different peptide pools. Many of the pools investigated were able to limit the virus spread when the cells were treated after infection with HSV-1. However, most of the pools were toxic for the Vero cells to different extents when incubated over 48 h. Since at the present stage of our research the single peptides from the pools with antiherpetic activity have not yet been isolated, it remains unclear whether antiviral activity and toxic effect are due to the same molecule or to two separate entities.

The pools derived from the proteolytical digestion of -lactalbumin, -lactoglobulin and lysozyme were treated with 3-hydroxyphthalic anhydride and their antiherpetic activity was investigated [118]. Most of the 3-HP modified pools showed anti-HSV-1 activity when present after the infection of the Vero cells, and several of them were able to block the virus multiplication upon treatment during infection. Moreover, some pools, which did not show antiviral activity, acquired antiherpetic properties after 3-HP modification. In contrast, some other pools lost their antiviral activity when treated with 3-HP, indicating that the modification of the lysine residues by 3-HP cannot be considered a general rule to increase the antiviral activity of peptides. In addition, all the 3-HP-pools examined were toxic for Vero cells to different extents.

CONCLUDING REMARK

The results presented in this review show that some food proteins possess antiviral properties. At present, such a property can be ascribed to only a few of them probably because investigations on this topic have been performed in a limited extent. It would be desirable to put a major effort in this matter in the future. Most of the food proteins have been considered only under their nutritive aspect until now but broadening the spectrum of the investigations might reveal antiviral properties.

Recently, food proteins have attracted more attention in the biological and medical community not only because of their nutrient value but as substances which constantly supply the organisms with bioactive compounds. Antihypertensive peptides, such as angiotensin converting enzyme inhibitors have been isolated from milk, corn, and fish proteins. Peptides with opioid properties have been derived from proteins present in wheat gluten and casein. Peptides with antioxidant activity have been isolated from soybean proteins. Bioactive peptides with antinociceptive activity and anxiolytic effect have been extracted from enzymatically digested plant proteins [1]. The first nutrient with which we come in contact in our life is milk containing several proteinic substances with the function to protect us from pathogenic microorganisms. Maternal immunoglobulins as well as the bactericidal proteins such as lactoferrin, lysozyme and lactoperoxidase are just some examples.

Antiviral activity of native and modified food proteins or fragments thereof has been demonstrated against a number of DNA and RNA, enveloped and non-enveloped viruses, respectively [9, 10, 12, 29, 95, 96, 114]. Many attempts to understand the mechanisms of action have been conducted either based on assay conditions, on specific protein-protein- or antibody-binding experiments, or on biochemical and biophysical studies. Based on assay conditions seemingly many proteins act by binding to viral or cellular surface proteins, thus blocking the attachment of the viruses to their cell receptors or inhibiting the fusion and/or penetration process [10, 13, 14, 31]. However, in some cases intracellular virus replication processes were suggested to be concerned. Finally, some proteins may be able to act after the cells have been damaged by virus replication, allowing them to penetrate into the cells and act on progeny viruses, or to prevent the cell-to-cell spread. Nevertheless, a number of questions concerning the action mechanisms of the antiviral proteins remain unanswered.

Although the presence of bioactive proteins as component of the diet is known since several years, only recently a great attention has been put on their potential therapeutic use. In this context, several bioactive peptides derived from food proteins have been isolated, characterized and their biological function investigated. It could be shown that they possess a large number of biological properties, from antimicrobial and immunostimulating activity to improving learning performance in mice [1]. Food proteins and their derivatives have the advantage, compared to synthetic pharmacologic compounds, to be non-toxic and surely well accepted by consumers' associations. Almost all food proteins investigated for antimicrobial activity originate from milk and chicken egg. This is not surprising because proteins derived from these two nutrients are commercially available in a highly purified grade, markedly simplifying the research on their biological properties. It would be of

great interest to search whether proteins with antiviral activity or more generally with antimicrobial properties are contained in other nutrients like meat, fruits and cereals.

Of particular interest is that some food proteins, like -lactalbumin or -lactoglobulin, without antiviral activity can be modified, through easy chemical reaction, to get antiviral properties. Most of the modified food proteins investigated were 3-HP modified proteins or acylated proteins. It would be noteworthy to investigate other structural modifications able to convert an inactive protein into one with antiviral activity. At present, we do not know which requirements are necessary for a protein to acquire antiviral properties. Extensive investigations are in progress to understand which physico-chemical characteristics are necessary to a protein or a peptide to get antibacterial properties [119]. Nothing similar, to our knowledge, is in progress for antiviral proteins. Further and deeper investigation on this area would be highly profitable.

Virus infection is still fought by vaccination. Unfortunately, vaccines against many of the important pathogenic viruses are not available. Chemically prepared compounds with antiviral activity might significantly contribute to decrease the incidence of disease transmission. The chemical modifications necessary to convert -lactoglobulin or other food proteins into anti-HIV compounds are easy and inexpensive. Therefore, they could be produced in large scale also in the developing countries contributing to the prevention of HIV transmission or other viral diseases.

The presented data show that food proteins can be tailored by proteolytical enzymes present in the gastrointestinal tract and generate in this way short peptide fragments with antiviral activity. This topic has been until now investigated only poorly and it would be desirable to intensify studies on this field. Short antiviral peptides can be synthesized in large amount with low production costs and antigenicity, which consequently limits possible hyper-sensitivity.

ABBREVIATIONS

3-HP = 3-Hydroxyphthalic anhydride

PIV-3 = Bovine parainfluenza virus type 3

Aco = *Cis*-aconitic anhydrid

AZT = Azidothymidine

CC_{50} = Cytotoxic concentration (Concentration of the compound which reduces the cell viability by 50%)

EC_{50} = Effective concentration (Concentration of the compound which reduces the virus yield by 50%)

EHV-1 = Equine herpes virus type 1

HCMV = Human cytomegalovirus

HCV = Hepatitis C virus

HIV-1 = Human immunodeficiency virus

HSV-1 = Human herpes simplex virus type 1

HSV-2 = Human herpes simplex virus type 2

MCMV = Murine cytomegalovirus

PRCV = Porcine respiratory corona virus

Suc = Succinic anhydride

REFERENCES

[1] Bioactive Peptides from Food Proteins; Pellegrini, A. Ed. Bentham Sci. Publ., **2003**, *Curr. Pharm. Des.* Vol. *9,* Nr 16.

[2] Bellamy, W.; Takase, M.; Yamauchi, K.; Wakabayashi, H.; Kawase, K.; Tomita, M. *Biochim. Biophys. Acta* **1992**, *1121*, 130 .

[3] Pellegrini, A. *Curr. Pharm. Des*. **2003**, *9*, 1225.

[4] Clare, D.A.; Catignani, G.L.; Swaigsgood, *Curr. Pharm. Des.* **2003**, *9*, 1239.

[5] Patick, A.K.; Potts, K.E. *Clin. Microbiol. Rev.* **1998**, *11*, 614.

[6] Hasegawa, K.; Motsuchi, W.; Tanaka, S.; Dosako, S. *Jpn J. Med. Sci. Biol.* **1994**, *47*, 73.

[7] Harmsen, M.C.; Swart, P.J.; de Béthune, M.P. ; Pauwels, R. ; De Clercq, E.; The, T.H. ; Meijer, D.K.F. *J. Infect. Dis.* **1995**, *172*, 380.

[8] Marchetti, M.; Longhi, C.; Conte, M.P.; Pisani, S.; Valenti, P.; Seganti, L. *Antiviral Res.* **1996**, *29*, 221.

[9] van der Strate, B.W.A.; Beljaars, L.; Molena, G.; Harmsen, M.C.; Meijer, D.K.F. *Antiviral Res.* **2001**, *52*, 225.

[10] Neurath, R.; Debnath, A.K.; Strick, N.; Li, Y.Y.; Lin, K.; Jiang, S; *J. Mol. Recogn*. **1995**, *8*, 304 .

[11] Debnath, A.K.; Jiang, S.; Strick, N.; Lin, K.; Haberfield, P.; Neurath, A.R. *J. Med. Chem.* **1994**, *37*, 1099.

[12] Neurath, R.; Li, Y.Y.; Strick, N.; Jiang, S. *Lancet*, **1996**, *347*, 1703.

[13] Berkhout, B.; van Wamel, J.L.B. ; Beljaars, L.; Meijer, D.K.F.;Visser, A.; Floris, R. *Antiviral Res*. **2002**, *55*, 341.

[14] Berkhout, B.; Derksen, G.C.H.; Back, N.T.K.; Klavier, B.; De Kruif, C.G.; Visser, S. *AIDS Res. Hum. Retrovir*. **1997**, *13*, 1101 .

[15] Swart, P.J.; Kuippers, M.E.; Smit, C.; Pauwels, R.; De Béthune, M.P.; De Clercq, E.; Meijer, D.K.F.; Huisman, J.G. *Aids Res. Hum. Retrovir.* **1996,** *12*, 769.

[16] Schoen, P.; Corver, J.; Meijer, D.K.F.; Wilschut, J.; Swart, P.J. *Biochem. Pharmac*. **1997**, *53*, 995.

[17] Baba, M.; Pauwels, P.; Balzarini, J. ; Arnout, J. ; Desmyter, J. ; De Clercq, E. *Proc. Natl. Acad. Sci. USA* **1988***, 85*, 6132.

[18] Mitsuya, H.; Looney, D.J.; Kuno, S.; Ueno, R.; Wong-Staal, F.; Broder, S. *Science* **1988**, *240*, 646.

[19] Lederman, S.; Gulick, R.; Chess, L. *J. Immunol.* **1989**, *143*, 1149.

[20] Parish, C.R.; Low, L.; Warren, S.H.; Cunningham, A.L. *J. Immunol*. **1990**, *145*, 1188 .

[21] Brew, K.; Grobler, J.A. In *-lactalbumin.* Advanced Dairy Chemistry, Elsevier Applied Science **1992**; Vol. *1,* pp 191-229.

[22] McKenzie F.H.; White, F.H., Jr. *Adv. Prot. Chem.* **1991**, *41*, 173.

[23] Hakansson, A.; Zhivotovsky, B.; Orrenius, S.; Sabharwal, H.; Svanborg, C. *Proc. Natl. Acad. Sci.* **1995**, *92*, 8064.

[24] Hakansson, A.; Svensson, M.; Mossberg, A.K.; Sabharwal, H.; Linse, S.; Lazou, I.; Lönnerdal, B.; Svanborg C. *Mol. Microbiol*. **2000**, *35*, 589.

[25] Pellegrini, A.; Thomas, U.; Bramaz, N.; Hunziker, P.; von Fellenberg, R. *Biochim. Biophys. Acta*, **1999**, *1426*, 439.

[26] Permyakov, E.A.; Berliner, L.J. *FEBS Letters*, **2000**, *473*, 269.

[27] Swart, P.J.; Kuippers, M.E.; Smit, C.; Pauwels, R.; De Béthune, M.P.; De Clercq, E.; Meijer, D.K.F.; Huisman, J.G. *Aids Res. Hum. Retrovir.* **1996,** *12*, 769.

[28] Arnold, D.; Di Biase, A.M.; Marchetti, M.; Pietrantoni, A.; Valenti, P.; Seganti, L.; Superti, F. *Antiviral Res.* **2002**, *53,* 153.

[29] Marchetti, M.; Superti, F.; Ammendolia, M.G.; Rossi, P.; Valenti, P.; Seganti, L. *Med. Microbiol. Immunol*. **1999**, *187*, 199.

[30] Superti, F.; Ammendolia, M.G.; Valenti, P.; Seganti, L. *Med. Microbiol. Immunol.* **1997***, 186,* 83.

[31] Oeverman, A.; Engels, M.; Thomas, U.; Pellegrini, A. *Antiviral Res.* **2003**, *59,* 23.

[32] Spear, P.G; Longnecker, R. *J. Virol.* **2003**, *77*, 10179 .

[33] Hambling, S. McAlpine, A.S., Sawyer, L. In *-lactoglobulin.* Advanced Dairy Chemistry, Elsevier Applied Science **1992**; Vol. *1,* pp. 141-190.

[34] Perez, M.D.; Calvo, M. *J. Dairy Sci*. **1995**, *78*, 978-988.

[35] Sawyer, L.; Kontopidis, G. *Biochim. Biophys. Acta* **2000**, *1482*, 136-148.

[36] Godovac-Zimmermann, J. *TIBS* **1988**, *13*, 64-66.

[37] Flower, D.R.; North, A.C.T.; Sansom, C.E. *Biochim. Biophys. Acta* **2000**, *1482*, 9-24.

[38] Akerstrom, B.; Flower, D.R.; Salier, J. P. *Biochim. Biophys. Acta* **2000,** *1482*, 1-8.

[39] Godova-Zimmermann, J; Conti, A.; Liberatori, J.; Braunnitzer, G. *Biol.Chem. Hoppe-Seyler*, **1985**, *366*, 431.

[40] Papiz, M.Z.; Sawyer, L.; Eliopoulos, E.E.; North, A.C.T.; Findlay, J.B.C.; Sivaprasadarao, R.; Jones, T.A.; Newcomer, M.E.; Kraulis, P.J. *Nature* **1986**, *324*,383 .

[41] Said, H.M.; Ong, D.E.; Shingleton, J.L. *Am. J. Clin. Nutr.* **1989**, *49*, 690.

[42] Pérez, M.D.; Diaz de Villegas, C. ; Sanchez, L. ; Aranda, P. ; Ena, J.M. ; Calvo, M. *J. Biochem*. **1989,** *106,* 1094 .

[43] Kokuba, H.; Aurelian, L.; Neurath, A.R. *Antivir. Chem. Chemother.* **1998**, *9*, 353.

[44] Ikeda, M.; Nozaki, A.; Sugiyama, K.; Tanaka, T.; Naganuma, A.; Tanaka, K.; Sekihara, H.; Shimotohno, K.; Saito, M.; Kato, N. *Virus Res.* **2000**, *66*, 51.

[45] Neurath, A.R.; Jiang, S.; Strick, N.; Lin, K.; Li, Y.Y.; Debnath, A.K. *Nature Medicine* **1996**, *2,* 230.

[46] Neurath, A.R.; Debnath, A.K.; Strick, N.; Li, Y.Y.; Jiang, S.; Antivir. *Chem.Chemother.* **1997**, *8*, 131.

[47] Neurath, A.R.; Strick, N.; Li, Y.Y. *Antivir. Chem. Chemother.* **1998**, *9*, 177.

[48] Neurath, A.R.; Debnath, A.K.; Strick, N.; Li, Y.Y.; Lin, K.; Jiang, S. *Antivir. Chem.Chemother.* **1997**, *8,* 141.

[49] Jiang, S.; Li, Y.Y.; Lin, K.; Strick, N.; Neurath, A.R. *Vaccines* **1997**, *97,* 327.

[50] Jiang, S.; Lin, K.; Strick, N.; Li, Y.Y.; Neurath, A.R. *J. Acquir. Immun. Defic. Syndr. Hum. Retrovir.* **1996**, *15*, 461.

[51] Zeder-Lutz, G.; Neurath, A.R.; van Regenmortel, M.H.V. *Biologicals* **1999**, *27*, 29.

[52] Swart, P.J.; Harmsen, M.C.; Kuipers, M.E.; Van Dijk, A.A.; Van Der Strate, B.W.A.; Van Berkel, P.H.C.; Nuijens, J.H.; Smit, C.; Witvrouw, M.; De Clercq, E.; De Béthune, M.P.; Pauwels, R.; Meijer, D. K.F. *J. Pep. Sci*. **1999**, *5*, 563.

[53] Wyand, M.S.; Manson, K.H.; Miller, C.J.; Neurath, R. *Antimicr. Agen. Chemother*. **1999**, *43*, 978 .

[54] Fleming, A. *Proc. Roy. Soc. Ser*. **1922**, *B 93*, 306.

[55] Spitznagel, J.K. In Regulation of Leucocytes Function. Contemporary Topics in Immunology. Snyderman E. Ed. New York Plenum **1984**, Vol. *14*, pp. 283-343 .

[56] Jollès, P.; Jollès, J. *Mol. Cell. Biochem.* **1984,** *63,* 165.

[57] Torbeck, R.L. ; Prieur, D.J. *Am.J. Vet. Res.* **1979**, *40*, 1531.

[58] Schindler, M.; Assaf, Y.; Sharon, N.; Chipman, D.M.; *Biochemistry*, **1977**, *16*, 423 .

[59] Hasselberger, F.X. Use of Enzymes and Immobilized Enzyme, Nelson-Hall Inc. Chicago **1978**; pp. 128.

[60] Osserman, E.F.; Klockars, M.; Halper, J.; Fishel, R.E.; In Lysozyme , Osserman, E.F.; Canfield, R.E.; Beychok, S. Eds. Academic Press, New York **1974,** pp. 471-490.

[61] Thacore, H.; Willet, H.P. *Am. Rev. Resp. Dis*. **1966**, *93*, 786.

[62] Lemarbre, P. ; Rinehart, J.J. ; Kay, N.E. ; Vesella, R.; Jacobs, H.S. *Blood* **1981**, *58*, 994.

[63] Sava, G. ; Ceschia, V. ; Zabucchi, G. *Eur. J. Caner Clin. Oncol*. **1988,** *24*, 1737.

[64] Posse, E.; De Arcuri, B.F.; Morero, R.D. *Biochim. Biophys. Acta* **1994**, *1193*, 101 .

[65] Qasba, P.K.; Kumar, S. *Critic. Rev. Biochem. Mol. Biol*. **1997**, *32*, 255.

[66] Pellegrini, A.; Grob, K.; von Fellenberg, R. *Letters in Appl. Microbiol*. **1990**, *10*, 201.

[67] Wild, P.; Gabrieli, A.; Schraner, E.M.; Pellegrini, A.; Thomas, U.; Frederick, P.M.; Stuart, M.C.A.; von Fellenberg, R. *Micr. Res. Thech*., **1997**, *39*, 297.

[68] Pellegrini, A.; Thomas, U.; Wild, P.; Schraner, E.M.; von Fellenberg R. *Microbiol. Res*. **2000**, *155*, 69.

[69] Laible, N. J.; Germaine, G.R. *Infect. Immun*. **1985,** *48*, 720.

[70] Pellegrini, A.; Thomas, U.; von Fellenberg, R.; Wild, P. *J. Appl. Bacter*. **1992**, *72,* 180.

[71] Ibrahim, H.R.; Higashiguchi, S.; Juneja, L.R.; Kim, M.; Yamamoto, T. *J Agric. Food Chem.* **1996**, *44*,1416.

[72] Lee-Huang, S.; Huang. P.L.; Sun, Y.; Huang, P.L.; Kung, H.F.; Blithe, D.L.; Chen, H.C. *Proc. Natl. Acad. Sci*. **1999**, *96*, 2678.

[73] Steinrauf, L.K.; Shiuan, D.; Yang, W.J.; Chiang, M.Y. *Biochem. Biophys. Res. Commun.* **1999**, *266*, 366 .

[74] Lampis, G.; Deidda, D.; Pinza, M.; Pompei, R. *Antivir. Chem. Chemother*. **2001,** *12,* 125.

[75] Cisani, G.; Varaldo, P.E.; Inganni, A.; Pompei, R.; Satta, G. *Curr. Microbiol*. **1984,** *10*, 35.

[76] Cisani, G.; Varaldo, P.E.; Pompei, R.; Valisena,S.; Satta, G. *Microbios* **1989**, *59* 73.

[77] Ibrahim, R.H.; Aoki, T.; Pellegrini, A. *Curr. Pharm. Des*. **2002**, *8,* 671 .

[78] Ibrahim, H.R.; Kato, A.; Kobayashi, K.; *J. Agric. Food Chem.* **1991**, *39*, 2077 .

[79] Ibrahim, H.R.; Yamada,M.; Matsushida, K.; Kobayashi, K.; Kato, A. *J. Biol.Chem.* **1994**, *269*, 5059.

[80] Brock, J. *Immunol. Today* **1995**, *16,* 417.

[81] Harrington, J.P. *Int. J. Biochem*. **1992**, *24,* 275.

[82] Lönnerdal, B.; Keen, C.L.; Hurley, L.S. *Am. J. Clin. Nutr.* **1985**, *41*, 550 .

[83] Lönnerdal, B.; Iyer, S. *Annu. Rev. Nutr.* **1995,** *15*, 93..

[84] Neville, M.C.; Catfield, K.; Hansen, L.; Lewis, A.; Monks, J.; Nuijens, J.; Ollivier-Bousquet, M.; Schanbacher, F.; Sawicki, V.; Zhang, P. *Adv. Exp. Med. Biol*. **1998**, *443*, 141 .

[85] Brock, J.H. *Biochem. Cell Biology* **2002**, *80*, 1.

[86] Wakabayashi, H.; Takase, M.; Tomita, M *Curr. Pharm. Des.* **2003**, *9*, 1277.

[87] Marchetti, M.; Pisani, S.; Antonini, G.; Valenti, P.; Seganti, L.; Orsi, N. *Biometals,* **1998**, *11*, 89.

[88] Valenti, P.; Marchetti, M.; Superti, F.; Amendolia, M.G.; Puddu, P.; Gessani, S.; Borghi, P.; Belardelli, F.; Antonini, G.; Seganti, L. *Adv. Exp. Med. Biol*. **1998**, *443*, 199.

[89] Fujihara, T.; Hayashi, K. *Arch. Virol.* **1995,** *140*, 1469.

[90] Puddu, P.; Borghi, P.; Gessani, S.; Valenti, P.; Belardelli, F.; Seganti, L. *Int. J. Biochem. & Cell Biol.* **1998**, *30*, 1055 .

[91] Swart, P.J.; Kuipers, E.M.; Smit, C.; van der Strate, B.W.A.; Harmsen, M.C.; Meijer, D.K.F. Adv. *Exp. Med. Biol.* **1998**, *443*, 205.

[92] Ikeda, M.; Sugiyama, K.; Tanaka, T.; Tanaka, K.; Sekihara, H.; Shimotohno, K.; Kato, N. *Biochem. Biophys. Res. Commun*. **1998**, *24,* 549.

[93] Nozaki,A.; Ikeda, M.; Naganuma, A.; Nakamura, T.; Inudoh, M.; Tanaka, K.; Kato, N. *J. Biol. Chem.* **2003**, *278*, 10162.

[94] Tanaka, K.; Ikeda, M.; Nozaki, A.; Kato, N.; Tsuda, H.; Saito, S.; Sekihara, H. *Jpn. J. Cancer. Res*. **1999**, *90*, 367.

[95] Superti, F.; Ammendolia, M.G.; Valenti, P.; Seganti, L. *Med. Microbiol. Immunol.* **1997**, *186*, 83.

[96] Superti, F.; Siciliano, R.; Rega, B.; Giansanti, F.; Valenti, P.; Antonini, G. *Biochem. Biophys. Acta* **2001**, *1528,* 107.

[97] Murphy, M.E.; Kariwa, H.; Mizutani, T.; Yoshimatsu, K.; Arikawa, J.; Takashima, I. *Arch. Virol*. **2000**, *145*, 1571.

[98] Murphy, M.E.; Kariwa, H.; Mizutani, T.; Tanabe, H.; Yoshimatsu, K.; Arikawa, J.; Takashima, I. *J. Vet. Med. Sci*. **2001**, *63*, 637.

[99] Lin, T.Y.; Chu, C. ; Chiu, C.H. *J. Infec. Dis*. **2002**, *186*, 1161.

[100] Marchetti, M.; Superti, F.; Ammendolia, M.G.; Rossi, P.; Valenti, P.; Seganti, L. *Med. Microbiol. Immunol*. **1999**, *187*, 199.

[101] Arnold, D.; Di Biase, A.M.; Marchetti, M.; Pietrantoni, A.; Valenti, P.; Seganti, L.; Superti, F. *Antiviral Res.* **2002**, *53*, 153.

[102] Di Biase, A.M. ; Pietrantoni, A.; Tinari, A.; Siciliano, R.; Valenti, P.; Antonini, G.; Seganti, L,; Superti, F. *J. Med. Virol***. 2003**, *69*, 495 .

[103] Tomita, M.; Yamauchi, K.; Teraguchi, S.; Hayasawa, H. In *Advances in Lactoferrin Research;* Spik *et al.*, Eds ; Plenum Press New York, **1998**; pp.198-195.

[104] Van Hooijdonk, A.C.M.; Kussendrager, K.D.; Steijns, J.M. *Brit. J. Nutr.* **2000**, *84* (Suppl. 1)*,* S127 .

[105] Swart, P.J.; Harmsen, M.C.; Kuipers, M.E.; Van Dijk, A.A.; Van Der Strate, B.W.A.; Van Berkel, P.H.C.; Nuijens, J.H.; Smit, C.; Witvrouw, M.; De Clercq, E.; De Béthune, M.P.; Pauwels, R.; Meijer, D.K.F. *J. Pep. Sci*. **1999**, *5*, 563.

[106] Schoen, P.; Corver, J.; Meijer, D.K.F.; Wilschut, J.; Swart, P.J. *Biochem. Pharmac.* **1997**, *53*, 995.

[107] Swaisgood, H.E. In *Chemistry of the Caseins.* Advanced Dairy Chemistry Elsevier Applied Science **1992**; Vol. *1,* pp. 63-110.

[108] Clare, D.A.; Catignani, G.L.; Swaigsgood, H.E. *Curr. Pharm. Des*. **2003***, 9,* 1239.

[109] Teschemacher, H. *Curr. Pharm. Des*. **2003**, *9*, 1331.
[110] Korhonen, H.; Pihlanto, A. *Curr. Pharm. Des*. **2003**, *9*, 1297.
[111] Meisel, H.; FitzGerald, R.J. *Curr. Pharm. Des*. **2003**, *9*, 1289.
[112] Yamamoto, N.; Ejiri, M; Mizuno, S. *Curr. Pharm. Des*. **2003**, *9,* 1345.
[113] Meisel, H. *Biopolymer*, **1997**, *43*, 119.
[114] Floris, R.; Recio, I.; Berkout, B.; Visser, S. *Curr. Pharm. Des*. **2003***,* *9*, 1257.
[115] Pellegrini, A.; Thomas, U.; Franchini, M.; Stöckli, M.; Klauser, S.; Hunziker, P.; von Fellenberg, R. *FEBS Letters*, **1994**, *344*, 261.
[116] Siciliano, R.; Rega, B.; Marchetti, M.; Seganti, L.; Antonini, G.; Valenti, P. *Biochem. Biophys. Res. Commun*. **1999**, *264*, 19.
[117] Superti, F.; Siciliano, R.; Rega, B.; Giansanti, F.; Valenti, P.; Antonini, G. *Biochim. Biophys. Acta* **2001**, *1528*, 107.
[118] Oevermann, A. Zur antiviralen Aktivität von natürlichen Proteinen und deren Fragmenten vor und nach chemischer Modifikation. Dissertation **2001**, University of Zürich, Switzerland..
[119] Antimicrobial Peptides: A New Class of Antibiotics; Pellegrini, A. Ed. Bentham Sci. Publ., *Curr. Pharm. Des.* **2002**; Vol. *8*, Nr 9.

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